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Rhizomorph Behaviour in *Armillaria mellea* (Vahl) Quél

II. Logistics of Infection

BY

S. D. GARRETT

(Botany School, University of Cambridge)

ABSTRACT

The growth of rhizomorphs of *Armillaria mellea* from small woody inocula through tubes of soil was characterized by a progressive decline in weekly growth increments. Initial growth rate was related to the size of the inoculum food-base; the subsequent decline is attributed partly to depletion of nutrient reserves in the inoculum through fungal respiration and growth, and partly to competition for nutrients between the main growing apex of the rhizomorph and its subordinate branch apices. The vigour of infection of potato tubers by rhizomorphs from such small woody inocula increased with size of the inoculum, and decreased with increasing distance between inoculum and tuber. When inoculum potential of *A. mellea* was low, the fungus was generally overtaken in the tuber tissues by soft-rotting bacteria, which prevented its further advance.

I. INTRODUCTION

IN modern discussions of the host-parasite relationship, it is comparatively rare to see the balance between host and parasite referred to as a 'struggle'. This expressive term, once commonly employed in this connexion, has now become somewhat old-fashioned, with increase in knowledge of the host-parasite relationship and the concurrent adoption of a more mechanistic outlook by plant pathologists. Nevertheless, although we nowadays are chary of using such animistic expressions in print, many of us doubtless continue to use them in our thinking about the host-parasite relationship. On the one hand, we have the attacking force, represented by the parasite; on the other the defence, represented by the resistance of the host plant, both passive and active. With root-infecting fungi, such as *Armillaria mellea*, invading by means of rhizomorphs, the temptation to use analogies from military strategy is particularly strong. The mycelium can be organized into rhizomorphs, representing a concentration of the attacking force; it can be redeployed in the form of fringing mycelium from the rhizomorphs, whereby invaded territory is exploited (Garrett, 1953). In specialized root-infecting fungi forming rhizomorphs, the infection habit is *ectotrophic*; rhizomorphs grow at first epiphytically over the surface of the host root, sending branches into the tissues as they grow along. Sometimes, as with *A. mellea*, rhizomorphs can also grow along the plane of weakness between bark and wood, instead of on the surface. Epiphytic growth over the surface of the host root is necessarily more rapid than growth through the internal tissues, where the rhizomorphs encounter the mechanical resistance of cell walls and the living resistance of

protoplasts. It is thus tempting to use here a military analogy and to view the ectotrophic growth habit as the nearest possible approach to attack by the fungus along a wide front, whereby the active defence forces of the host root are either thinly stretched, or even evaded altogether, as observations and experiments by various workers indeed suggest (Garrett, 1955).

This paper deals with an experimental inquiry into the logistics of infection by the rhizomorphs of *A. mellea*. Elucidation of the other side of the host-parasite relationship, that of the mechanism of host resistance, is equally important, but this has received more attention, having been studied in considerable detail by Thomas (1934). Observations over a period of years on a number of fungi infecting tree roots have established the conclusion that the size and condition of the infected root, or root fragment, acting as inoculum is critical for the establishment of a successful, i.e. progressive, infection. Ideas on this subject were notably clarified by Gadd (1936), who introduced into general use the term *food-base*, to express the function of the inoculum as a reserve of nutrients essential for the establishment of infection. It is now recognized, though from a plenitude of field observations rather than from actual experiments, that the distance of the fungal food-base from the host root to be infected may be as critical for the success of infection as the nutritional reserves at the disposal of the fungus in the food-base. Thus although the rhizomorphs of *A. mellea* can grow freely through the soil for a distance of many feet from a sufficiently substantial food-base, it has been doubted whether infection by such rhizomorphs can occur outside a radius of more than a few feet from the food-base (Wallace, 1935).

The purpose of the investigation described hereunder has been to clarify the relationships between size and distance away of the fungal food-base constituting the inoculum, and the infective potentialities of the fungus. Comprehensive consideration of these relationships is assisted by the concept of *inoculum potential*. This term has been used by various authors in a sense that has been quite consistent, but which has varied in inclusiveness from one user to another; as I have been able to find no definition of its usage, it is essential for me to define here the sense in which I shall employ the term inoculum potential, as I have done elsewhere for more general use (Garrett, 1955). *Inoculum potential is defined as the energy of growth of a parasite available for infection of a host, at the surface of the host organ to be infected.* Implicit in this definition of inoculum potential as the energy of growth of a parasite is the qualification of energy of growth *per unit area of host surface*, without which any such definition would be meaningless. Inoculum potential of a parasite can be increased in either or both of two ways: (a) by an increase in the number of infecting units or propagules of the parasite per unit area of host surface, (b) by an increase in the nutritional status of such units. The habit of mycelial aggregation into rhizomorphs automatically secures, *ipso facto*, an increase in inoculum potential, through the maximum possible concentration of individual hyphae at the point of invasion. Since inoculum potential has been defined as the energy of growth of a parasite *at the surface*

of the host, it expresses the infective potentialities of the growing tips of the rhizomorphs, or of their branches, at the time of penetration of the host. The objective of the present investigation can thus be more concisely stated as follows: to determine the effect of size and distance away of the inoculum food-base upon the inoculum potential of rhizomorphs.

No previous investigation of this type with *A. mellea* seems to have been reported. The effect of inoculum characteristics, including size, upon infection of tree roots by various fungi has been examined (summarized in Garrett, 1944, 1955), but experimental data concerning the effect of inoculum distance upon infection seem to be lacking. This may be attributed in part to the fact that experiments with fungi infecting tree roots in their natural habitats are very laborious, and may take several years to complete. For this reason, a more rapid method of laboratory experimentation has been developed, and this has served at least for a preliminary exploration of the problem. Detached potato tubers have proved satisfactory as host organs in lieu of tree roots; although the resistance of potato tubers to infection by *A. mellea* is certainly lower than that of large tree roots, yet it is still sufficient for the purpose of experiment, and it has been possible to scale down inoculum weights and distances so as to give a satisfactory range in infection behaviour of the rhizomorphs. Individual potato tubers of a suitable size (100–80 g. fresh weight) can conveniently be accommodated within 2-lb. glass jam jars filled with moist sand or soil, and were previously found useful for experiments with another root-infecting fungus, *Helicobasidium purpureum* (Garrett, 1946).

II. METHODS

Woody inocula of *A. mellea* were prepared as follows. Shoots not exceeding 1.75 cm. diameter at the base were cut from pollarded willows (*Salix alba*) and stripped of their branches before being cut serially into 1 in. segments (segments of length 2 in. were used for Inoculation Experiment 1, in which segments of the maximum weight, 9 g., had to be accommodated within glass tubes of 1.9 cm. diameter). The segments were weighed and separated into weight classes, according to the requirements of the particular experiments that had been planned. Sufficient segments of the same weight class to occupy the bottom of a conical flask (250 or 500 ml.) were covered with maize-meal-sand culture medium (in the proportions 100 g. dry sand: 3 g. maize-meal: 15 ml. water). After autoclaving for 1 hr. at $1\frac{1}{2}$ atm. (22 lb.) pressure, flasks were inoculated with agar disks cut from a growing colony of *A. mellea*, and then incubated for $2\frac{1}{2}$ months at 25° C. before use. After this period of incubation at 25° C., flasks were stored until required in a dark cupboard at laboratory temperature; inoculum was still satisfactory for rhizomorph production after a further period of $3\frac{1}{2}$ months' storage (the maximum period tested) at laboratory temperature.

The soil employed throughout these experiments was a light loam from my own Cambridge garden, and was chosen for its favourable growth charac-

teristics, and for its ease of handling in the laboratory; its moisture-holding capacity (hereafter designated by 'm.h.c.') was determined by the perforated box method as 44 ml. water per 100 g. air-dry soil. Both for growth studies and for inoculation experiments with rhizomorphs, the soil was contained in glass tubes of internal diameter 1.9 cm., and length 25 or 38 cm. (c. 10 or 15 in.) according to the requirements of the particular experiment. These tubes were filled with air-dry soil in short sections (2.5 or 5 cm.), to which the required amount of water was then added from a graduated pipette; soil moisture content was usually adjusted to 60 per cent. m.h.c. Until the moistened soil had set in position, the open lower end of the tube was closed by a cottonwool plug; this was preferable to a rubber bung, because it permitted free passage of the air displaced by the downwards percolation of the soil water. By moistening the soil in short sections not exceeding 5 cm. in length, a rapid equilibration of soil moisture content was obtained (originally checked by determinations of soil moisture content on successive layers of the moist soil). For growth studies of rhizomorphs, the woody inoculum segments were placed either in the middle of the soil column, at the centre of the tube, so that growth could be measured both upwards and downwards, or else at the upper end of the soil column, when growth was measured downwards. In the latter instance, the inoculum segment was covered with moist sand, as an additional precaution against drying; rhizomorphs do not grow out into sand (Growth Experiment 1), so that this covering prevented dissipation of inoculum reserves by growth of rhizomorphs in any other direction than downwards through the soil column. When thus set up and inoculated, the soil tubes were capped at each end with lacquered, moisture-proof 'Cellophane', secured by a rubber band, and the weight of each tube was recorded on the label before placing in the 25° C. incubator, in which all experiments on growth of rhizomorphs were maintained. The 'Cellophane' caps were very effective in preventing moisture loss; this rarely exceeded 2 g. over a period of 6 weeks, after which time it could be restored by addition of water on a balance. At this time, the caps were replaced by fresh ones, because some mould growth often occurred on the 'Cellophane', especially if scattered particles of soil had fallen on to the bottom cap.

Visible growth of rhizomorphs from the inoculum into the soil column usually occurred within 2–3 weeks at 25° C. The first growth measurement was made when the majority of tubes in an experiment showed young rhizomorphs; thereafter, weekly measurements were made of the *maximum extent* of rhizomorph growth from the inoculum in each tube. Since some of the rhizomorphs were hidden within the soil column, and none was visible except at intervals when it grew alongside the glass wall of the tube, this was the only method that could be adopted with this particular technique of observation. The error entailed by this method was offset by making measurements on a minimum of ten replicates in all except the first experiment. The degree of precision was also increased by the method of expressing the final results. In these experiments, interest centred more on the trend in mean weekly

growth rate than on the total extent of linear growth. For each tube, therefore, weekly increments of growth were calculated, starting from the first weekly measurement at which any rhizomorph growth was recorded. In this way, it was possible to eliminate variation due to the fact that rhizomorphs were initiated from some inoculum segments earlier than from others; in a small proportion of tubes, initiation of rhizomorphs was more considerably delayed, though only rarely did it fail to occur within the period of an experiment.

For Experiments 1 and 3 on inoculation of potato tubers, inoculum segments of different weight classes were set up at the upper ends of soil columns of different lengths, as described above; in Experiment 3, soil columns of different lengths were inoculated at intervals, so as to reduce, as far as was practicable, the variation in time at which rhizomorphs reached the ends of their soil tubes. Potato tubers (variety Majestic) for inoculation were selected for uniformity of size and shape, and for freedom from wounds and blemishes. In order to eliminate error due to a possible relation between tuber size and resistance to infection (which, however, a subsequent statistical examination of the results failed to substantiate), the four replicate tubers allotted to each experimental series were chosen, with due randomizing precautions, so as to cover approximately the same weight range as those of all the other series. In setting up the inoculation trials, each tuber was placed at the bottom of an empty 2-lb. glass jam jar. The inoculum tube (with the lower 'Cellophane' cap removed and moist soil brought flush with the end of the tube) was then placed with its lower end resting upon a plane surface of the tuber, and as close to the centre as possible. With the inoculum tube held firmly in position, 750 g. dry sand was poured around tuber and inoculum tube, levelled off, and moistened to 50 per cent. m.h.c. by addition of 75 ml. distilled water. The inoculum tube was thereafter held firmly in position by the moist sand. Each jar was labelled with its own weight; jars were then arranged in randomized blocks upon a laboratory table, and kept under a frame covered by light-proof paper. Once a week, each jar was weighed and loss through evaporation was restored by addition of water on a box balance. During the latter part of each experiment, it became necessary to remove the potato sprouts, which were cut off as they appeared above sand level. The precise method of recording infection data varied with individual experiments, and will be recorded in its context below.

III. EXPERIMENTS ON GROWTH OF RHIZOMORPHS THROUGH TUBES OF SOIL

Experiment 1. A small trial was set up to determine the optimum soil moisture content for growth of rhizomorphs in the particular soil employed; a comparison was made between tubes of soil adjusted to moisture contents of 40, 60, and 80 per cent. m.h.c. Earlier observations had indicated that rhizomorphs are not produced by woody inocula buried in sand; some

initials may be produced, but none of them makes appreciable growth through the sand. One series of tubes was therefore filled with quartz sand at 60 per cent. m.h.c., and a second series with a mixture of one volume soil to three volumes sand, adjusted to the same proportional moisture content. After tubes had been filled, inoculum segments of weight 3 g. were placed at the tops of soil columns and covered with moist sand. Maximum downwards growth of rhizomorphs (means of six replicate tubes) after 28 days at 25° C. is given in Table I.

TABLE I
Maximum Extent of Rhizomorph Growth (mm.)

Soil, 80% m.h.c.	Soil, 60% m.h.c.	Soil, 40% m.h.c.	Soil/sand	Sand
67	66	61	18	0.5
Standard error = 3.88				

There was no significant difference in rhizomorph growth at these different soil moisture contents, but a moisture content of 60 per cent. m.h.c. has been selected as a standard for all subsequent experiments. Growth of rhizomorphs into sand was negligible, and was confined to a growth of 3 mm. in $\frac{1}{8}$ tubes. This observation that growth of rhizomorphs into quartz sand rarely occurs, and then only to a negligible extent, has been repeatedly confirmed throughout this investigation. This effect cannot be due to a toxicity; it has occurred with all samples of quartz sand tested, none of which has given any signs of toxicity either to micro-organisms or to higher plants. It seems that soil provides some stimulus to production and outwards growth of rhizomorphs that is lacking in sand; thus the soil/sand mixture containing $\frac{1}{4}$ soil by volume produced a quite consistent growth of rhizomorphs, though smaller than that in undiluted soil. In subsequent experiments, moist sand has proved very useful as a covering for inoculum segments placed at the tops of soil columns, since it has restricted growth of rhizomorphs in any direction except downwards through the soil column, as desired.

Experiment 2. Weekly increments of rhizomorph growth were compared for inoculum segments of weights 4, 2, 1, and 0.5 g. Columns of soil at 60 per cent. m.h.c. were set up in sixty tubes, thus giving fifteen replicate tubes for each inoculum weight series. Inoculum segments were placed at the tops of soil columns and covered with moist sand. Results are given in Table II.

These data show a progressive and significant decline ($P < 0.001$) in weekly growth increments over the 5-week period, and a progressive and significant reduction ($P < 0.001$) in size of growth increments with reduction in size of inoculum. Nevertheless, the rate of decrease in weekly growth increments over the 5-week period does not seem to be determined by the total bulk of nutrient reserves in the inoculum segment, because if lines are drawn on a graph through points representing weekly growth increments, their slopes are found to be roughly parallel for all inoculum weights. The

fact that rate of reduction in weekly growth increments is independent of inoculum size can also be demonstrated through the analysis of variance performed on the above data, which shows no significant interaction between inoculum weight and reduction in weekly growth increments (variance ratio for interaction/error = 0.46).

TABLE II
Successive Weekly Growth Increments of Rhizomorphs (mm.)

		Weekly periods					Mean (± 1.01)
		1	2	3	4	5	
Inoculum weight	4 g.	25	24	20	16	12	16
	2 g.	23	21	19	15	14	15
	1 g.	17	15	13	10	10	11
	0.5 g.	16	14	14	10	4	9
Mean (± 1.13)		20	19	17	13	10	
Standard error for individual means = 2.27.							

Reduction in weekly growth increments of rhizomorphs could be attributed to some factor of the soil environment, such as soil aeration, varying down the soil column. If, however, a deterioration in soil aeration had been responsible for the slowing down of growth, it could be expected that growth would have accelerated once rhizomorphs had passed the mid-way points of the columns. Weekly growth measurements were continued beyond those given

TABLE III
Successive Weekly Growth Increments of Rhizomorphs (mm.)

		Weekly periods					Mean (± 1.31)
		1	2	3	4	5	
Inoculum weight	4 g.	25	23	17	16	8	18
	2 g.	20	20	13	12	7	14
	1 g.	18	12	13	9	5	12
Mean (± 1.69)		21	18	14	12	7	
Standard error for individual means = 2.92							

in Table 2, and showed no indication of this; the decline in weekly growth increments continued after rhizomorphs had passed the mid-way points of the soil columns. Any such postulated effect of soil aeration, moreover, would have worked in the opposite way in the next experiment, in which inoculum segments were placed at the mid-points of soil columns.

Experiment 3. Inoculum segments of three weight classes, 4, 2, and 1 g., were buried at the mid-points of 30 cm. soil columns. Five replicate tubes were allotted to each inoculum weight series, giving a replication of ten growth measurements, i.e. one up and one down from the inoculum segment in each tube. The results of the first series of measurements are given in Table III.

Once more, the progressive decline in weekly growth increments over the 5-week period is significant ($P < 0.001$), and so is the reduction in growth

increments with decrease in inoculum weight ($P = 0.01$). An analysis of variance again shows no significant interaction between inoculum weight and rate of decline in weekly growth increments over the 5-week period (variance ratio for interaction/error = 0.52).

This conclusion that the occurrence of a progressive decline in weekly growth increments of rhizomorphs is independent of inoculum size was then tested in another way. As soon as the growth measurements obtained for Table III had been completed, after a total incubation period of $7\frac{1}{2}$ weeks, all inoculum segments were taken from their tubes, and their attached rhizomorphs were removed. The segments were then set up in fresh columns of soil, arranged exactly as before, and a second series of growth measurements was obtained (Table IV).

TABLE IV
Successive Weekly Growth Increments of Rhizomorphs (mm.)

		Weekly periods					Mean (± 1.19)
		1	2	3	4	5	
Inoculum weight	4 g.	18	13	13	7	6	11
	2 g.	20	14	11	8	4	11
	1 g.	14	9	9	4	6	9
Mean (± 1.54)		17	12	11	6	5	
Standard error for individual means = 2.67							

It can be seen from Table IV that removal of rhizomorphs from inoculum segments was followed by the production of new rhizomorphs, which at first grew more quickly than the older ones that they replaced. Initial growth rates were lower than those recorded in Table III, however. In fresh inoculum segments, the level of initial rhizomorph growth rate must be conditioned by the rate at which nutrients can be made available; this is related to size of inoculum segments, as shown in Table III. During the period of $7\frac{1}{2}$ weeks for which the inoculum segments remained in the soil during growth of the first crop of rhizomorphs, total nutrient reserves of the inocula were diminished by fungal respiration and growth; in consequence, initial growth rates of the second crop of rhizomorphs, though still related to inoculum size, were at a generally lower level.

Nevertheless, although gradual consumption of the food reserves in the inoculum accounts for part of the observed decline in rhizomorph growth rate, it does not account for the whole of it; if it did, then the initial growth rates of the second crop of rhizomorphs would be no higher than the final observed growth rates of the older rhizomorphs that they replaced. It is not difficult to account for a gradual decline in rhizomorph growth rate at a constant level of nutrient supply from the inoculum. Indeed, a constant level of nutrient supply from the inoculum does not entail a constant supply of nutrients to the main growing apices of the rhizomorphs. What has actually been measured in these observations has been the maximum rhizomorph growth rate, i.e. the rate of growth of the leading rhizomorph. When this

particular rhizomorph grows out from the inoculum segment, it is at first unbranched, but later it may branch freely; the nutrient supply at first wholly available to the main apex must later be shared amongst an increasing number of branch apices, and hence the supply to the main apex must progressively decline. A similar decline in rate of nutrient supply to the main apex of the leading rhizomorph may also be brought about by the subsequent growth of other and younger rhizomorphs from the inoculum segment, if rhizomorphs happen to be produced over an interval of time, rather than all together at the outset of growth. Counts made of rhizomorph numbers at times of the first and fourth weekly growth increments showed an increase from six to seventeen rhizomorphs per 4 g. inoculum segment, but no significant increase in numbers for 2 g. inoculum segments. The nutrient supply to the main growing apex may also be affected by other factors, such as the efficiency of translocation along the lengthening rhizomorph.

IV. EXPERIMENTS ON INFECTION OF POTATO TUBERS

Experiment 1. In this first experiment, it was necessary to guess at the ranges of inoculum size and distance likely to show a suitable range of infection behaviour. Five inoculum weights (9, 7, 5, 3, and 1 g.) were combined factorially with four inoculum distances (2.5, 5, 10, and 15 cm.), with a replication of four. The soil tubes, with inoculum segments placed at tops of soil columns and covered with moist sand, were incubated at 25° C., to start rhizomorph growth as quickly as possible; an initial period of 12 days at 25° C. was given to the 2.5 and 5 cm. soil column tubes, and one of 20 days to the 10 and 15 cm. tubes. After this, tubes were placed in position above the potato tubers to be inoculated, as described under 'Methods' above. Allowance had to be made for the fact that rhizomorphs would reach the potato tubers later after traversing the longer soil columns; the tubers were therefore left in the jars after inoculation for 3 months, a period that should have allowed ample time for the development of maximum possible infection even in the 15 cm. soil column series. When this time was up, all tubers were examined for extent of infection. Some penetration by *A. mellea* had occurred in every one of the eighty tubers included in this experiment, but the extent of infection varied considerably from one series to another. To demonstrate this, tubers were grouped into two classes: (a) those showing 100 per cent. infection of the tissue by *A. mellea*, (b) those showing less than 100 per cent. infection. The number of tubers, out of a total of four replicates in each series, showing 100 per cent. infection is given in Table V.

The number of tubers with 100 per cent. infection declined both with decrease in inoculum weight and with increase in inoculum distance. A criticism of the validity of this conclusion concerning the effect of inoculum distance could be based on the fact that rhizomorphs growing through 15 cm. soil had less time in which to occupy the whole of the tuber than had rhizomorphs growing through only 2.5 cm. soil, inasmuch as all inoculum segments

were started at the same time. This criticism is discounted by the following observation. During examination of the tubers, it was noted that where infection of a tuber by *A. mellea* was incomplete, the remainder of the tissue had generally been occupied by soft-rotting bacteria. *A. mellea* could have

TABLE V
Number of Tubers with 100 per cent. Infection by A. mellea
(out of 4 replicates)

		Inoculum distance				Mean
		2.5 cm.	5 cm.	10 cm.	15 cm.	
Inoculum weight	9 g.	4	4	3	1	3.0
	7 g.	4	4	3	1	3.0
	5 g.	4	4	1	2	2.75
	3 g.	3	3	1	0	1.75
	1 g.	2	3	0	0	1.25
Mean		3.4	3.6	1.6	0.8	

made no further progress in these tubers, for however long they had been left (Table VI).

A reasonable explanation of these results can be suggested. Where inoculum potential of *A. mellea* had been high (large inocula, small distances), the fungus was quickly able to overcome host resistance and thus to invade the whole of the tuber with little delay. But where inoculum potential of *A.*

TABLE VI
Number of Tubers in which further progress by A. mellea had been arrested by
development of Bacterial Soft Rot (out of 4 replicates)

		Inoculum distance				Mean
		2.5 cm.	5 cm.	10 cm.	15 cm.	
Inoculum weight	9 g.	0	0	0	2	0.5
	7 g.	0	0	0	2	0.5
	5 g.	0	0	3	2	1.25
	3 g.	1	0	3	3	1.75
	1 g.	0	0	4	3	1.75
Mean		0.2	0	2	2.4	

mellea had been low (small inocula, greater distances), a prolonged struggle between *A. mellea* and the active resistance of the tuber occurred, until further progress of the fungus was arrested through secondary invasion by soft-rot bacteria.

Experiment 2. An attempt was next made to determine the minimum weight of inoculum effective in securing infection of potato tubers by *A. mellea*, when placed in close proximity to the tuber. Inoculum segments of weight 2, 1, 0.5, and 0.25 g. were separated from the potato tubers, which were buried as usual in jars of moist sand at 50 per cent. m.h.c., by a short column of soil, 1 cm. long and 2.4 cm. diameter. This soil column was included within the

sand in order to stimulate rhizomorph production by the inoculum; a previous experiment had shown that infection of potato tubers in sand was irregular and uncertain, even when inocula were placed in direct contact with the tubers. Tubers were examined for extent of infection by *A. mellea* after 6½ weeks; degree of infection was expressed both by fresh weight of infected tissue and by radial extent of infection (Table VII: figures are means from nine replicate tubers).

The effect of inoculum weight upon infection was significant ($P < 0.001$) by both methods of estimation. No infection occurred from the 0.25 g. inoculum segments, but only one out of nine had produced any rhizomorphs; this single segment produced one short rhizomorph, which had remained epiphytic upon the surface of the tuber.

TABLE VII

Effect of Inoculum Size on Vigour of A. mellea in Infection

		Fresh weight of infected tissue (g.)	Radial extent of infection (mm.)
Inoculum weight	2 g.	10	16
	1 g.	4	11
	0.5 g.	0.2	3
	0.25 g.	0	0
Standard errors		1.72	2.53

The infected tubers obtained in this experiment provided good material for study of the early stages of infection. Penetration of the tuber tissue was by means of rhizomorph branches given off by the epiphytic rhizomorphs running over the surface; on one such rhizomorph, five branches were counted over an interval of 6 mm. In section, these young branch rhizomorphs were sometimes irregular in shape and frequently somewhat constricted at the point of penetration of the tuber periderm. Once within the tuber, the rhizomorphs developed into expanded white sheets of aggregated mycelium, which Thomas (1934) has termed 'internal rhizomorphs'. These observations on penetration of potato tubers closely confirmed those made by Thomas in his study of penetration of potato tubers, carrots, parsnips and woody tree roots by rhizomorph branches of *A. mellea*.

Experiment 3. An attempt was made to improve upon Experiment 1 in two respects. The range of inoculum distances was extended from a minimum of 5 cm., by 5 cm. steps, to 25 cm. The five inoculum distances were combined in a factorial design with three inoculum weights, 4, 2, and 1 g., making fifteen series in fourfold replication. Secondly, an endeavour was made to remove a flaw in the design of Experiment 1, in which rhizomorphs from the more remote inocula must have reached the tubers later than those from nearer inocula. If the resistance of the potato tubers increased with age, or with length of incubation in moist sand, this arrangement of the

experiment could have biased the results; although there is no particular evidence for such a supposition, it was obviously desirable to remove this possible source of error. In the present experiment, therefore, the 25 cm. soil column tubes were prepared and inoculated first, and placed in the 25° C. incubator; the 20-cm. column tubes were set up and placed in the incubator a fortnight later, and were followed at fortnightly intervals by the 15-, 10-, and 5-cm. column tubes. Three weeks after the 5-cm. column tubes had been placed in the incubator, all tubes were removed thence, and used for inoculation of the sixty potato tubers comprising the experiment. A fortnight at 25° C. was not sufficient to enable rhizomorphs to grow the necessary distance of 5 cm. in the later stages of growth, and hence to bring rhizomorphs to the ends of tubes in all series at the time of inoculation of potato tubers.¹ Nevertheless this arrangement at least reduced substantially the discrepancies that would have resulted had all inocula been started together, as in Experiment 1, and subsequent statistical examination of the results has failed to reveal any bias in degree of infection attributable to this cause (see below, Table X).

Tubers were removed from their jars, for recording of infection by *A. mellea*, 15½ weeks after inoculation, so that ample time would have been allowed for infection even by the later arriving rhizomorphs. Eight out of the sixty tubers had to be discarded from the experiment, because rhizomorphs failed to reach them. These were in the following inoculum weight/inoculum distance series: one replicate each of '4 g.-15 cm.' and '1 g.-25 cm.'; two replicates each of '4 g.-25 cm.', '2 g.-25 cm.', and '1 g.-15 cm.' The long period of incubation allowed for this experiment, for reasons just given, solved one problem only by creating another—that of assessing the extent of infection. The fresh weight of infected tissue is directly related to rate of advance of the fungus only if the infected tissue is increasing in the form of a complete, expanding sphere. For this reason, the direct measurement of radial extent of infection, as employed in Experiment 2 (Table VII), must always be a more reliable criterion than the indirect measurement, made by assessing the fresh weight of infected tissue. The error thus inherent in the fresh-weight method of estimation must increase as infection proceeds, and as it becomes increasingly limited by the restrictions of tuber size and shape. This difficulty was recognized by Gregory and Horne (1928, *a* and *b*), in their work on fungal rotting of apple fruits. By making the limited and reasonable assumption that the apple fruit could be regarded as a sphere for the practical purpose of their calculations, Gregory and Horne were able to derive a relationship between fresh weight of infected tissue and rate of 'radial advance' of the fungus, and thus to make excellent use of a mass of data already obtained by weighing determinations. Owing to the irregularity in shape of the potato tuber, the method of Gregory and Horne could not be used here. It was also too late to make direct measurements of the radial extent of

¹ This experiment had already been set up before the final results of Growth Experiment 2 had been calculated, and so no allowance had been made for the unsuspected decline in rhizomorph growth rate.

infection, as had been done in Experiment 2, because in many of the tubers the fungus had already reached the farthest part of the perimeter.

From these considerations, it seemed inadmissible to use the methods of recording infection that had been appropriate for Experiment 2 under the circumstances of the present experiment, in which the precision of the measurements would be inconsistent with the degree of significance that could be attached to them. The same kind of objection, however, could not be sustained to the use of a more crude method designed to record relatively gross differences in the extent of infection, because such a method would make no pretensions to a degree of accuracy that it did not possess. Preliminary inspection of a number of tubers, before any recording was done,

TABLE VIII

Estimated Percentage Volume of Tissue occupied by A. mellea in Tuber

		Inoculum distance					Mean (± 2.09)
		5 cm.	10 cm.	15 cm.	20 cm.	25 cm.	
Inoculum weight	4 g.	93	91	93	88	40	81
	2 g.	81	85	80	28	28	60
	1 g.	73	78	58	28	4	48
Mean (± 2.70)		82	85	77	48	24	

Standard error for individual means = 4.67

showed considerable differences in the extent of infection, and suggested that tubers could be classified by eye according to the percentage volume occupied by *A. mellea*. Each tuber was therefore sliced up, examined thoroughly, and classified in one of the following groups for percentage volume of tissue occupied by *A. mellea*: 100, 95, 90, then by steps of 10 down to 10, 5, and 1 per cent. (Table VIII).

The degree of infection by *A. mellea* has decreased significantly both with decrease in inoculum size ($P < 0.001$) and with increase in inoculum distance ($P < 0.001$). The minimum inoculum distance effective in reducing infection decreased with decrease in inoculum size, indicating an interaction between the factors of inoculum size and inoculum distance in their effect upon inoculum potential. Analysis of variance has shown this interaction to be highly significant ($P < 0.001$).

The observations of Experiment 1 with respect to the development of bacterial soft rots were confirmed. Incomplete infections by *A. mellea* had usually been overtaken by bacterial soft rots, which were sometimes still advancing into healthy tissue at the time of examination, and had clearly originated in the lesions originally made by *A. mellea* (Table IX).

Amongst the eight tubers in the experiment that were not inoculated with *A. mellea*, because rhizomorphs failed to reach them, two spontaneously developed bacterial soft rots. Therefore the occurrence of more than one

tuber out of four with bacterial soft rot in Table IX can be attributed to predisposition through a host-contested primary infection by *A. mellea*. Total absence of bacterial soft rot, on the other hand, in any series of four tubers shown in Table IX (i.e. for 4 and 2 g. inocula at distances of 5–15 cm.) can be ascribed to rapid occupation of the tissues by *A. mellea* before bacterial soft rots were able to develop spontaneously.

Finally, an analysis of the data on infection by *A. mellea* was made in order to determine whether the degree of infection could have been influenced by variation in the time at which rhizomorphs reached the tuber; although the

TABLE IX

Number of Tubers in which further progress by A. mellea had been arrested by development of Bacterial Soft Rot (out of 4 replicates)

		Inoculum distance					Mean
		5 cm.	10 cm.	15 cm.	20 cm.	25 cm.	
Inoculum weight	4 g.	0	0	0	1	2	0.6
	2 g.	0	0	0	2	4	1.2
	1 g.	1	1	2	4	2.7 ¹	2.1
Mean		0.3	0.3	0.7	2.3	2.9	

¹ Fractional value calculated from 3 tubers

delay was greater at the greater inoculum distances, the four replicates in any one series also showed considerable variation in this respect. A record had been kept of the 'rhizomorph growth deficit', i.e. the distance between apical portions of rhizomorphs and the end of the soil tube, for each tube at time of inoculation of the potato tuber. Appreciable deficits occurred only in the

TABLE X

Percentage Infection of Tubers by A. mellea in relation to Rhizomorph Growth Deficits

	Rhizomorph growth deficit groups			
	I	II	III	IV
Mean rhizomorph growth deficit (cm.)	4.5	8.2	9.2	11.0
Mean percentage infection of tuber	52	22	29	46

20 and 25 cm. inoculum distance series, within each of which were contained three inoculum weight series. Each of the four replicate tubers in each of these six inoculum weight/inoculum distance series was therefore placed in one of four groups, according to the degree of rhizomorph growth deficit with which it had been associated. Mean percentage infection of the tubers in each of the four rhizomorph growth-deficit groups was then calculated (Table X).

Since there was clearly no significant difference between mean percentage

infection of tubers in group IV, with a rhizomorph growth deficit of 11.0 cm., and that in group I, with a deficit of only 4.5 cm., it is concluded that variation in rhizomorph growth deficit has not appreciably biased the results given in Table VIII.

V. DISCUSSION

It remains to relate the results of these laboratory experiments to the behaviour of *Armillaria mellea* in the field, so far as this is known. The dependence of infection upon a food-base of adequate size has already been demonstrated for several root-infecting fungi, and the experiments with *A. mellea* described here confirm this conclusion. The proportion of successful, i.e. progressive, infections declines not only with decreasing size of food-base, but also with increasing distance of the food-base from the host organ to be infected. It is not difficult to account in general terms for this observed effect of distance, but it is difficult to discriminate experimentally between a number of alternative though not mutually exclusive hypotheses. From field observations, it has been concluded by several authors that infection by *A. mellea* occurs either through direct contact between infected and healthy roots (Dade, 1927), or through the agency of rhizomorphs over a very limited distance (Wallace, 1935). This limiting effect of distance upon infection by rhizomorphs can be explained in several ways. A buried root or other piece of timber infected by *A. mellea* eventually sends out rhizomorphs in all directions. The farther away a healthy root is from this inoculum, the smaller is the chance that a rhizomorph will encounter the root during its outward growth from the inoculum, and the smaller will be the number of rhizomorphs that eventually impinge upon unit length of root. This effect of distance in causing a radial dispersal of rhizomorphs as they recede from the inoculum has been eliminated in these laboratory experiments by canalizing the growth of all rhizomorphs put out from an inoculum segment, through confinement within a glass tube. Nevertheless, an increase in distance away of the inoculum has still produced a similar effect to that of decrease in size of the inoculum, i.e. there has been a decrease in inoculum potential of the fungus, resulting in a slower rate of invasion of the host tissues. Statistical examination of the results has demonstrated a highly significant interaction ($P < 0.001$) between these two variables, inoculum size and inoculum distance.

Observations on rate of growth of rhizomorphs from woody inoculum segments of various sizes through glass tubes filled with soil have shown a progressive decline in the size of weekly growth increments. They have further demonstrated that this decline in growth rate can be analysed into two components: (a) a decline ascribed to decrease in nutrient-supplying power of the inoculum with lapse of time, through gradual exhaustion of nutrients by fungal respiration and growth, (b) a decline in growth rate of the main growing apex under conditions of constant nutrient supply from the inoculum, which has been tentatively attributed to competition for nutrients

by subordinate branch apices and sometimes, perhaps, to competition from other, later-developing rhizomorphs.

This decline in growth rate of the main apex of a rhizomorph with increasing distance from its food-base represents a decrease in the available energy of fungal growth at the apical growing point, or, in other words, a decrease of inoculum potential, as it has been defined above, and this could conceivably account for the observed results of the inoculation experiments. It is necessary to remember, however, that a number of rhizomorphs are put out by each inoculum segment; with increasing distance from the inoculum, the interval of time between arrival of successive rhizomorphs at the surface of the host is likely to increase and this would certainly reduce the initial impetus of fungal invasion. Further experiments on infection, by inocula from which growth is restricted to a single rhizomorph, are now required.

VI. SUMMARY

Rhizomorphs of *Armillaria mellea* grow out in all directions from woody inocula within a few weeks of burial in moist soil, but no outwards growth of rhizomorphs occurs if inocula are buried in quartz sand. Successive weekly growth increments have been calculated for rhizomorphs growing out from inocula of various sizes into glass tubes filled with soil. Initial growth rate depends upon the size of the inoculum; a subsequent progressive decline in growth rate has been attributed to (a) gradual consumption of nutrient reserves in the inoculum through fungal respiration and growth, (b) increasing competition for available nutrients between the main growing apex of the rhizomorph and its subordinate branch apices. Similarly, late-formed rhizomorphs could compete for nutrients with those first put out from the inoculum.

The relation of these observations to the results of inoculation experiments with *A. mellea* on potato tubers is discussed. Speed of infection by rhizomorphs of *A. mellea* increased with increase in size of the inoculum, and decreased with increasing distance between inoculum and potato tuber. Statistical examination of the data showed a highly significant interaction ($P < 0.001$) between the effects of these two variables, size and distance away of the inoculum, upon infection. When inoculum potential of *A. mellea* was low, advance of the fungus into the tuber tissues was slowed down by host resistance; such delayed infections were generally overtaken by soft-rotting bacteria, which occupied the remainder of the potato tuber, and effectively halted any further advance by *A. mellea*.

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The Life-History and Reproduction of *Polyides caprinus* (Gunn.) Papenf.

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With Plate VI and six Figures in the Text

ABSTRACT

This paper deals with the development of the sexual organs (in their respective nemathecia), the carposporophyte and the tetrasporophyte of *Polyides caprinus* in the British Isles. The carposporophyte is of the 'diffuse type' and resembles that of some other Cryptonemiales and some Gigartinales (*sensu* Kylin) in essential features. The life-history is of the *Polysiphonia*-type on the coast of Wales, as male, female, and tetrasporic plants occur in nature and reduction division takes place in the tetrasporangium.

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I. INTRODUCTION

POLYIDES CAPRINUS is the only species of this genus which was included by Schmitz and Hauptfleisch (1897) in the family Rhizophyllidaceae. Later Kylin (1944) moved the genus to the new family the Polyideaceae without giving any diagnostic family characters, leaving this new family, as well as the older one, in the Cryptonemiales. The family contains the two genera *Polyides* and *Rhodopeltis*, and the distinguishing characters appear to be based on those of the former and the better-known genus.

P. caprinus is confined to the colder latitudes of the northern hemisphere and occurs commonly in the inter-tidal rock-pools of many shores of the British Isles. It is a dark-purple or purple-red lithophyte of cartilaginous texture when fresh, but becomes horny and black when dry. The repeatedly

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dichotomously branched upright cylindrical fronds arise from a perennial disk of varying size. Growth of the thallus is brought about by a number of apical cells and the mature thallus is differentiated into a 'medulla', an inner and an outer 'cortex'.

In the British Isles, male, female, and tetrasporic thalli occur and so there is no reason to doubt that the life-history is of the *Polysiphonia*-type. On the other hand, the preponderance of sexual plants of *P. caprinus* in Denmark (Rosenvinge, 1917), the absence of tetrasporic plants on the west coast of Sweden (Kylin, 1923), and the occurrence of tetrasporangia on sexual individuals (Sinova, 1912) are suggestive of a deviation from the *Polysiphonia*-type of life-history.

Although there have been several investigations of carposporophytes of species belonging to the Cryptonemiales, comparatively few are as detailed as is desirable. Least is known of the nemathecial types. Our existing knowledge of the development of the sexual organs and carposporophyte of *P. caprinus* dates back to the classical researches of Thuret and Bornet (1878), subsequent investigators including Rosenvinge (1917) and Kylin (1923) having contributed little. The carposporophyte appears, on the basis of the available information, to be of the type general in the Cryptonemiales. Excepting the Corallinaceae, the carposporophyte of the Cryptonemiales conforms in general to a uniform pattern, being of the 'diffuse type'. One or more gonimoblasts develop either directly from the fertilized carpogonium or from certain cells of the carpogonial branch with which the fertilized carpogonium has previously communicated. The auxiliary cell branches are well separated from the fertile branches.

The only cytological investigations of any extent in this large Order are those of Yamanouchi (1921) on *Corallina officinalis* var. *mediterranea* and Suneson (1950) on species of *Lithophyllum*.

For these reasons an investigation of the life-history and reproduction of *Polyides caprinus* seemed desirable.

2. MATERIAL AND METHODS

Most of the material of *P. caprinus* used in this investigation has been collected at frequent intervals over a period of one year from Rhosneigr on the west coast of Anglesey Island (North Wales), but in addition valuable collections have been received from other parts of the United Kingdom.

Plants required for cytological observations have been fixed on the shore, only such plants as were completely submerged under water being used. Further material has also been brought to the laboratory and fixed at different times of the day. The maximum number of nuclei in division has been found in plants fixed on the shore between 12.30 p.m. and 2.30 p.m. when low water was approximately 3-4 hours later.

Material has been fixed in a variety of fixatives, but the best results have been given by formalin-alcohol (Drew, 1934) and formalin-acetic-alcohol

(Westbrook, 1928). Whatever be the type of fixative employed, the material has had to be softened before being squashed or sectioned. The method for softening described by Drew (1945) has been followed. Kylin's (1923) method for softening material has also been used for both fresh and fixed material to be stained subsequently with Gram's iodine solution.

Free-hand sections and serial microtome sections ($6-24\ \mu$ thick) have been employed in the study of nemathecium and carposporophytes. Squash preparations (E. Greig-Smith, unpub.) have been found, however, to be very much more useful in studying meiosis in the tetrasporangium and the organization of the carposporophyte.

Out of a number of cytoplasmic and nuclear stains tried, Brazilin (Drew, unpub.), and Feulgen's reagent proved the best for cytological purposes and Gram's iodine for dissections of nemathecium used for studying the development and organization of the carposporophyte.

3. DEVELOPMENT OF THE SEXUAL REPRODUCTIVE ORGANS OF THE GAMETOPHYTE

Early stages in the development of male and female nemathecium, which then look alike, have been observed in plants collected during August and September. When first recognizable the nemathecium appear to the naked eye as small, pale yellow, pink or purple, oval or circular patches of different sizes. Transverse and longitudinal sections of such pieces of thalli reveal that nemathecium are formed from groups of apical cells of the cortex. (Text-fig. 1.) The number of cells concerned in the formation of each group and the position of the nemathecium on the thallus are both highly variable. Nothing is known about the factors responsible for the stimulation of the apical cells into active division, nor is any information available at present regarding the factors that control such a fortuitous development of these fertile tracts on the thallus. The cells which give rise to nemathecium are first recognizable by their greater size, large nuclei, and homogeneous protoplasm. The nucleus is near the basal wall and the cells divide in quick succession giving rise to short, compactly arranged parallel filaments composed of discoid cells which are broader than long (Text-fig. 1, B).

The development of the male and female nemathecium filaments differs slightly from this stage onwards. The distal cells of the former undergo periclinal divisions resulting in the divergence of the filaments. Large, fully developed male nemathecium may cover the entire surface of the upper dichotomies of the thalli.

While the male nemathecium filaments pierce through the cuticle and diverge at a very early stage, those of the female nemathecium grow and branch parallel for a while, lifting the cuticle ahead of them by their concerted growth. However, patches of the original cuticle of the thallus with numerous epiphytic diatoms attached may sometimes be seen included between the bases of the fully grown nemathecium filaments.

The cells of the filaments very soon become quadrate in section and contain a proportionately large nucleus in the lightly staining cytoplasm. When the sterile nemathecium filaments reach a length of about 10–12 cells, short side branches begin to develop, usually 3 or 4 cells below the apical cell. All the cells of the nemathecium filaments by now contain many small floridean starch grains. The increase in length of the nemathecium filaments is due to the activity of the apical cells and the elongation of the cells thus formed. Each nemathecium filament has its own transparent mucilaginous sheath, but at a later stage those parts covering the filament apices coalesce to give a continuous canopy-like covering which appears to toughen and thicken with age. In old nemathecium the lower surface of this covering shows a honeycomb-like



TEXT-FIG. 1. A. Part of a transverse section of a female plant showing the formation of nemathecium filaments. Some of the apical cells to the left are about to divide. B. A slightly later stage than A. The mucilaginous sheaths of individual filaments are visible at the apices. (Brazilin.) $\times 566$.

pattern (seen frequently in squashes) with the apices of filaments fitting into the circular or polygonal areas. Lateral extension of nemathecium takes place by the continued production of more nemathecium filaments at the margin of those already initiated. The centrifugal spreading of adjacent nemathecium may lead to their fusion and eventually to the formation of large nemathecium extending completely around the thallus. Ultimately when the carpospores have been shed nemathecium filaments are cast off.

All the filaments of male nemathecium are fertile and they grow to varying lengths before the cells undergo longitudinal divisions. Such divisions never occur in the basal 3 or 4 cells which elongate considerably. The proximal axial cells give rise to one or two mother-cells, while the distal ones form 3 or 4. Usually three, but sometimes one, two, or four spermatangia are borne by each mother-cell. The contents of the spermatangium are liberated as a single spherical colourless spermatium. The nucleus of the liberated spermatium is in prophase, and usually surrounded by a narrow zone of cytoplasm. The delicate, transparent wall of the liberated spermatium is clearly seen in preparations stained with cytoplasmic stains and in spermatia which are attached to trichogynes. The account of the development of spermatia is in close agreement with the accounts of Schmitz (1883) and Kylin (1923). Neither of these authors, however, describes the development

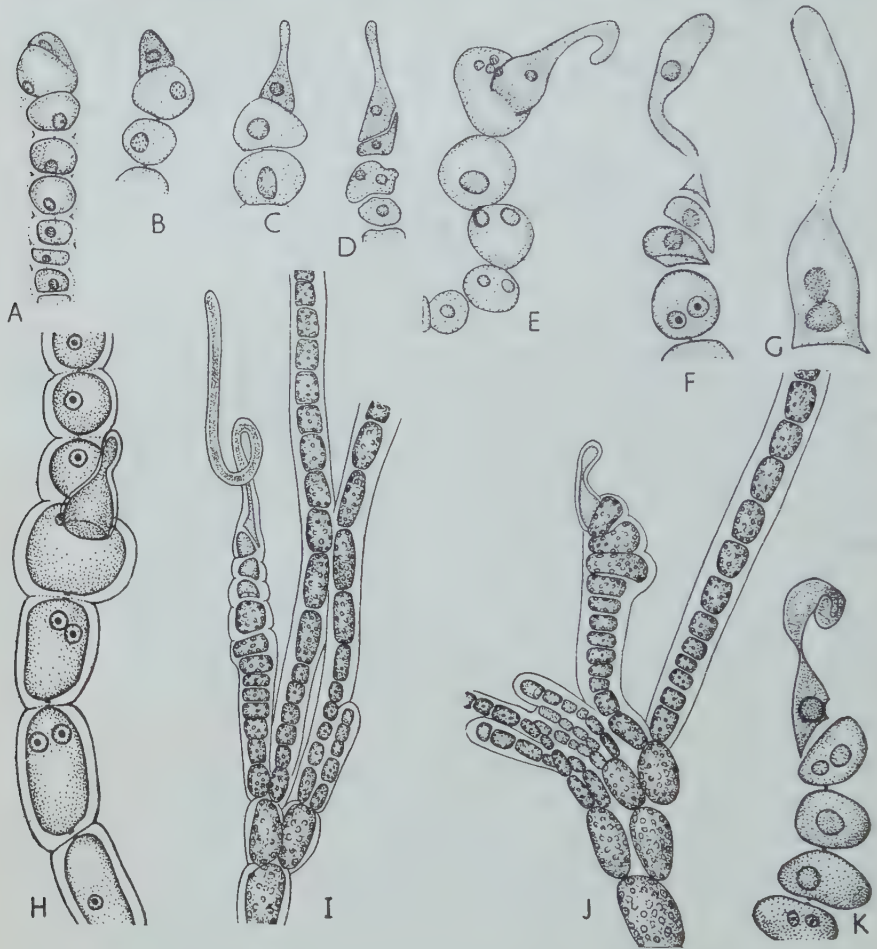
of such long brush-like branchlets as have been found during this investigation.

Only certain filaments of the female nemathecium bear carpogonia. When the sterile nemathecial filaments are ten to twelve cells long and the cells of some filaments begin to enlarge, certain other slightly shorter filaments become arrested in their growth. These shorter filaments function as fertile branches and bear carpogonia. Since they are slightly curved and the trichogynes are usually twisted, the entire branch is seldom obtained in a single section. In following the development of the fertile branch, therefore, use has been made not only of serial microtome sections but also of squash preparations as well as dissections stained with iodine.

The number of cells in a fertile branch is very variable, the earliest formed branches containing fewer cells than the later formed branches. As the production of carpogonia continues throughout the existence of nemathecium, all stages in their development can be seen even in nemathecium containing mature carposporophytes. The apical cell of the fertile branch divides by an oblique wall into a small upper cell, the carpogonium-initial, and a large lower one, the hypogynous cell (Text-fig. 2, A). Subsequently the apical region of the apical cell elongates into a trichogyne which at no stage in its development has been observed to possess a nucleus. In a few cases the trichogyne begins to develop before the carpogonium is separated from the hypogynous cell (Text-fig. 2, D). The carpogonia are therefore apical and sessile, but in some instances the wall between the carpogonium and hypogynous cell is so oblique that the carpogonium appears to be lateral. The mucilaginous sheath surrounding the carpogonium and trichogyne is a continuation of the sheath of the filament.

Carpogonia with forked trichogynes or two separate trichogynes have sometimes been observed. In a single example two carpogonia had arisen from the same hypogynous cell and hence one must have been lateral. Sometimes the development of the carpogonium appears to be arrested very early and it may then be transformed into a long or short hyaline hair. Since the carpogonia which are formed early in the development of the nemathecium terminate filaments almost as long as the sterile nemathecial filaments, a comparatively short trichogyne reaches to the outer surface. The later-formed carpogonia, however, are situated at varying distances from the surface of the nemathecium and the trichogynes of those which terminate short branches only reach the surface if very long. It appears likely, therefore, that only the earliest-formed carpogonia of the nemathecium with short trichogynes are fertilized. Moreover, their maturation synchronizes with the development of spermatangia. At the time of fertilization the trichogyne either just reaches the surface or projects slightly beyond. The cytoplasm stains deeply and a single small nucleus is to be seen in the basal region of the carpogonium. In the majority of instances spermatia have been seen attached to the apical gelatinized region of the trichogyne and rarely to its sides as figured by Thuret and Bornet (1878). By the time the gonimoblast is formed, spermatia

can no longer be seen still attached to the trichogyne as figured by Thuret and Bornet (1878), Pl. XXXVIII, Figs. 15, 16, and 18. This present investiga-



TEXT-FIG. 2. A. Carpogonium-initial formed by the oblique division of the apical cell of a fertile branch. All cells of the branch are uninucleate. B. Conical carpogonium-initial. C. Trichogyne slightly elongated. D. Carpogonium-initial divided to form the hypogynous cell after the trichogyne has elongated. E. Sessile lateral carpogonium; hypogynous cell four-nucleate. F. Carpogonium showing what is presumably the male nucleus in the trichogyne. G. Early stage of fusion of male and female nuclei within the carpogonium. H. Fertile branch showing an apparently lateral, sessile carpogonium due to formation of lateral branch from hypogynous cell. I and J. Fertile branches from old nematocia; in I the trichogyne has elongated and three cells below the carpogonium are depleted of floridean starch. J. Carpogonium nearly lateral and trichogyne not yet elongated. K. Fertilization nucleus. Neck of the trichogyne greatly constricted. (I and J. Iodine; E. Feulgen; the rest Brazilin.) G $\times 1700$; I and J $\times 425$; the rest $\times 850$.

tion has led the writer to question whether this may not be a composite figure therefore. The entry of the spermatium nucleus into the trichogyne has not

been seen. However, in a number of examples a deeply staining nucleus has been seen at various positions within the trichogyne. It is assumed that these are male nuclei on account of the close resemblance between the nuclei of the spermatia and such nuclei seen in trichogynes. (Pl. VI, Fig. 7.) In a few examples two small nuclei, presumably the male and female gametic nuclei, have been observed in the basal region of the carpogonium (Text-fig. 2, G, Pl. VI, Fig. 8). A deeply stained large nucleus filling most of the basal region of the carpogonium has been found in still other examples and it is presumed that the large nucleus represents the fertilization nucleus (Text-fig. 2, K). The trichogynes of what appear to be both fertilized and unfertilized carpogonia persist. The trichogyne becomes broad and contracted after fertilization and sometimes bent and coiled. The neck region of the trichogyne becomes greatly constricted (Text-fig. 2, K; Pl. VI, Fig. 8).

At the time the carpogonium is initiated all the cells of the fertile branch are uninucleate and full of floridean starch grains. At a later stage, a variable number of the cells in the upper part of the branch become swollen and protruberances develop. All the protruberances may be on one side or be arranged spirally. Frequently, one or more cells of the fertile branch bear one- or two-celled branchlets. In a few examples seen the hypogynous cell had branched, the carpogonium having been pushed to one side and thus appearing lateral. Meanwhile, the number of nuclei in the cells of the fertile branches increases to two, three, or four (Text-fig. 2, E).

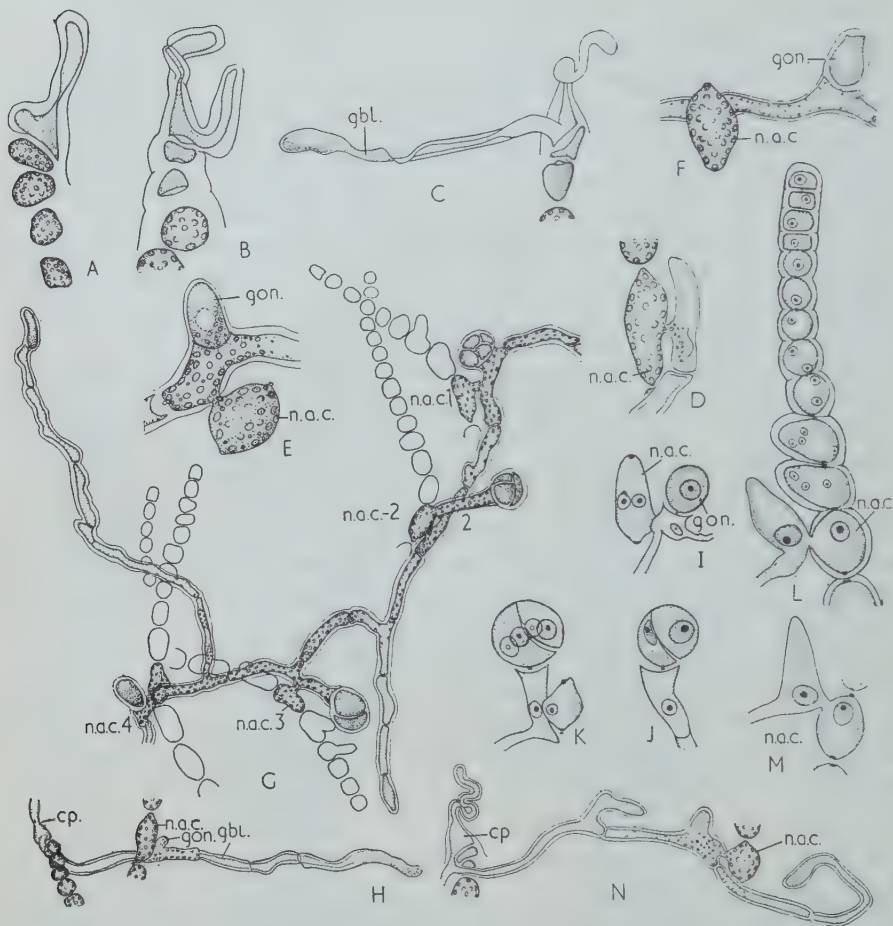
The cells of fertile branches bearing carpogonia with very elongated trichogynes are often small, shrivelled, and mis-shapen. In addition, the cytoplasm of as many as seven cells beneath the carpogonium is refringent and homogenous, floridean starch being absent. The disappearance of the starch starts in the hypogynous cell and progresses down the branch.

4. DEVELOPMENT OF THE CARPOSPOROPHYTE¹

The fertilized carpogonium gives rise direct and without fusion with any other cell of the fertile branch to a single broad outgrowth, the gonimoblast, which may be directed upwards, sideways, or downwards (Text-fig. 3, B-C). The gonimoblast elongates following an undulating course between the lower part of the closely-set nemathecial filaments. In consequence, it is difficult to follow it in sections and the following account is based mainly on squash preparations. At first the whole gonimoblast contains dense cytoplasm, but as it elongates, this is confined to the apical region only. The dark-staining nucleus is situated in this cytoplasm a short distance from the apex. (Pl. VI, Fig. 12.)

As the gonimoblast grows and branches, it fuses successively with a number of certain intercalary cells of some of the nemathecial filaments. These cells are indistinguishable from other cells of the nemathecial filaments and are more or less at the same distance from the surface as are the carpogonia. In spite

¹ The terminology used in this section is that proposed by Drew (1954).



TEXT-FIG. 3. A-C. Origin of the gonimoblast from fertilized carpogonia. Note that in A none of the cells of the fertile branch are depleted of floridean starch. D. Early stage in development of gonimolobe-initial after fusion of gonimoblast with nutritive auxiliary cell. E. Gonimolobe-initial formed from the gonimoblast in a lateral position. F. Gonimolobe-initial formed slightly away from the region of contact with the nutritive auxiliary cell. G. Part of a carposporophyte showing the fusion of one gonimoblast with four nutritive auxiliary cells (1-4). Contents of the auxiliary cells only are shown, although the other cells are also full of floridean starch. H. Well-developed gonimoblast showing connexion with the carpogonium. No fusions between the carpogonium and the cells of the fertile branch. Fusion of the gonimoblast with an auxiliary cell and the early appearance of a gonimolobe-initial seen. I-K. Three stages in the division of the gonimolobe-initial. L and M. Early stages in the development of gonimolobe-initials from the point of fusion of a gonimoblast with an auxiliary cell. Auxiliary cell uninucleate. N. Young gonimoblast still showing connexion with carpogonium, and fusion with a nutritive auxiliary cell and the formation of two branches, one near the carpogonium and the other between the carpogonium and the nutritive auxiliary cell.

cp, carpogonium; *gbl*, gonimoblast; *n.a.c.*, nutritive auxiliary cell; *gon.*, gonimolobe-initial.

(I-M, Brazilin; the rest iodine.) G, H, N $\times 285$; the rest $\times 566$.

of repeated efforts to find examples of the stages in fusion between a gonimoblast and such a cell, none have been seen. Being very delicate structures the earliest stages of fusion do not survive the pressure applied in squashing. Slightly more advanced stages showing open communication between the auxiliary cell and gonimoblast have sometimes been seen. In no example observed during this investigation has there been any indication of the gonimoblast nucleus entering the auxiliary cell (Pl. VI, Fig. 11). Using Drew's (1954) terminology, they are therefore nutritive auxiliary cells. After fusion with the first nutritive auxiliary cell, the gonimoblast grows on to fuse with other similar cells at varying distances from each other. As the gonimoblast lengthens it becomes considerably branched, increases in diameter, and septa can be clearly seen. Pit-connexions are visible in the transverse walls and the longitudinal walls are usually provided with short stumpy protrusions. The cells of the gonimoblast contain starchy food especially in the vicinity of the nutritive auxiliary cells. By contrast, the auxiliary cells lose their floridean starch (Text-fig. 3, G-M).

The formation of special spore-producing lateral branches of the gonimoblast is initiated by the fusion of the gonimoblast and the nutritive auxiliary cells. During its growth the gonimoblast fuses with a number of nutritive auxiliary cells, but whereas the formation of the spore-producing lateral branches is limited to the region of the fusions in the early stages of the development of the carposporophyte, this is not so in the later stages, the spore-producing laterals (the gonimolobes) being formed remote from nutritive auxiliary cells. The gonimolobes of any one carposporophyte therefore outnumber the nutritive auxiliary cells. These spore-forming lateral branches, each of which forms a gonimolobe, are formed from the gonimoblast and not the auxiliary cell in one of the following ways:

1. The tip of the main gonimoblast may be cut off as a gonimolobe-initial and a branch which continues the growth of the gonimoblast may arise just near the region of contact with the auxiliary cell (Text-fig. 3, G at 3).

2. A short branch may arise from the enlarged communication tube between the gonimoblast and the auxiliary cell and its tip is cut off as a gonimolobe-initial (Text-fig. 3, G at 2).

3. A gonimolobe-initial may terminate a short lateral branch of the main gonimoblast either near the place of fusion with the nutritive auxiliary cell or slightly farther away from it (Text-fig. 3, G at 4, and F).

The gonimolobe-initial contains homogeneous dense cytoplasm and a large nucleus (Text-fig. 3, 1). The initial enlarges and divides by successive walls into a large central cell and generally three or rarely four small peripheral cells separated by curved walls. The peripheral cells act as apical cells and divide by walls at right angles to the basal walls. The cells thus formed divide repeatedly until a spherical cluster of cells is formed. Floridean starch accumulates in all these cells and eventually the peripheral cells of the developing gonimolobe filaments enlarge and form obconical carposporangia.

The latter are generally uninucleate, but sometimes they may be two- or three-nucleate. A section of a mature gonimolobe shows the central cell surrounded by a number of small cells, the placenta, and a peripheral zone of carposporangia, the whole cluster being enclosed in a firm transparent mucilaginous membrane.

If several carpopogonia in a nemathecium are fertilized, a corresponding number of carposporophytes will develop, but even if several carposporophytes are present they cannot be distinguished individually.

As has been stated, the gonimoblast is formed without any prior fusion of the carpogonium with cells of the fertile branch. However, in twelve examples such fusions have been seen. They were found in nemathecia containing gonimolobes in an advanced stage of development and the evidence suggests that they should be regarded as secondary fusions occurring after the development of the gonimoblast. This interpretation is supported by the example shown in Text-fig. 4, A, in which a branch from the base of the gonimoblast has apparently grown down and fused with the third and fourth cells of the fertile branch. From the down-growing portion of the gonimoblast one branch has arisen subsequently. These secondary fusions appear to have taken place with those cells of the branch immediately beneath the depleted cells.

Another interesting example is shown in Text-fig. 4, B, and in this fusion has taken place with the third and fifth cells of the fertile branch. From the point of fusion with the fifth cell there appears to have arisen a branch which has given rise near by to a gonimolobe and then continued its growth. The stalk cell of the gonimolobe from which the spores have been liberated is seen as a dilated cell (*st*) in the figure. A comparison of the sizes of the fertile branches shown in Text-fig. 3, A–C and this example suggests that these are secondary fusions which take place late in the development of the gonimoblast.

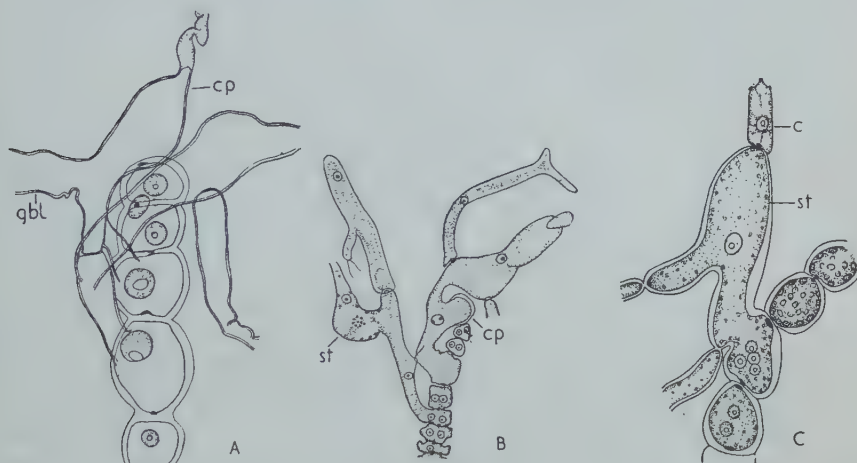
Thuret and Bornet (1878) considered that such fusions between the carpogonium and the cells of the branch below take place before the formation of the gonimoblast. The gonimoblast in their figure is wide, thick-walled, septate, and branched, a condition the present investigation has shown to be indicative of a late stage in development. It seems likely, therefore, that Thuret and Bornet did not see the earliest stages and interpreted the secondary fusions incorrectly, supposing them to have preceded the development of the gonimoblast.

During the development of the carposporophyte the auxiliary cell branch shows characteristic changes, particularly concerning the branching and number of nuclei per cell. As soon as a gonimoblast fuses with a nutritive auxiliary cell, the latter increases in size, usually becoming spherical or ovoid and its cytoplasm showing greater affinity for Brazilin and haematoxylin. The nucleus and pit-connexions also increase in size. Frequently the nucleus divides and gives rise to two, three, or four nuclei. Examples have been observed in which the auxiliary cell nucleus had divided into four daughter

nuclei, while the gonimolobe-initial was still uninucleate, and others in which the auxiliary cell showed a single enlarged nucleus at a very advanced stage of gonimolobe development.

Other cells of the auxiliary cell branch, especially the two or three cells lying immediately below and above the nutritive auxiliary cell, show changes similar to those that take place in the auxiliary cell fusing with the gonimoblast.

The large deeply stained uni- or multi-nucleate auxiliary cells with two or three neighbouring multi-nucleate cells with asymmetrical protrusions are



TEXT-FIG. 4. A and B. Secondary fusions of the gonimoblast with cells of the fertile branch. In A the gonimoblast has produced a downward growth which has fused with the third and fourth cells and another branch has arisen from the downward growth. Nucleus of gonimoblast with dark nucleolus and nuclei of gametophyte with white nucleoli. B. Fusions have taken place with the third and fifth cells of the fertile branch. The dilated cell is the stalk-cell of gonimolobe from which the cells have been severed. Most of the cells of the fertile branch are binucleate. Nuclei of gonimoblast indicated by dark nucleoli. C. Auxiliary cell branch from an old nemathecium showing the enlarged stalk-cell. Auxiliary cell four-nucleate and the cell below two-nucleate.

c, central cell; *cp*, carpagonium; *gbl*, gonimoblast; *st*, stalk-cell.

(A, Brazilin; B and C, iodine.) A $\times 566$; B $\times 166$; C $\times 285$.

conspicuous in stained squashes and sections (Pl. VI, Fig. 12). In old nemathecia containing mature carposporangia, the cells of auxiliary cell branches are larger than those of the neighbouring filaments and their pit-connexions are conspicuous, sometimes occupying the breadth of the septum. In stained sections of nemathecia such branches can easily be traced to the centre of the thallus. This fact indicates that the food materials pass to the gonimolobes via the nutritive auxiliary cell. In old auxiliary cells the region of contact with the gonimoblast becomes slightly elongated and in some instances the greatly enlarged stalk-cell of the gonimolobe has the appearance of a side process of the auxiliary cell (Text-fig. 4, c).

5. REDUCTION DIVISION IN THE TETRASPORANGIUM AND OTHER CYTOLOGICAL OBSERVATIONS

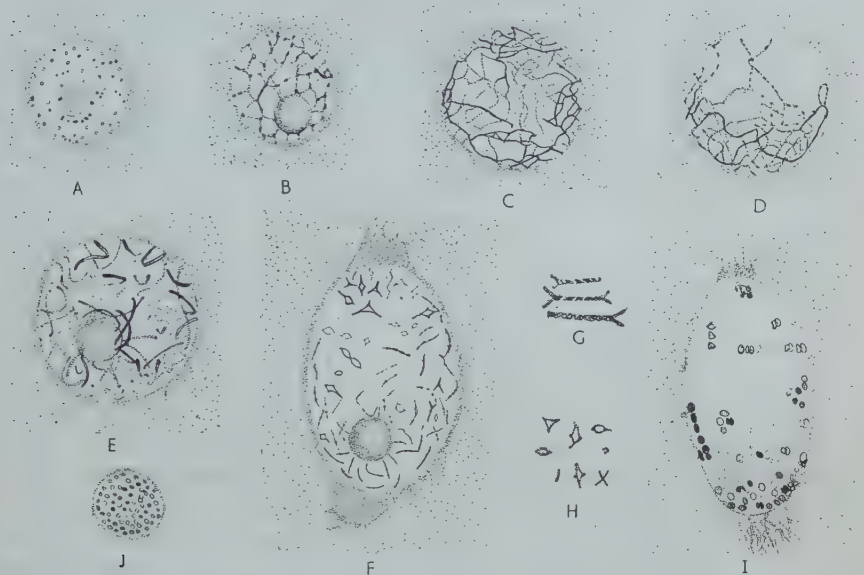
The following account is based on squash preparations stained with either Brazilin or Feulgen's reagent. For revealing the details of the structure of chromosomes in the early stages, Brazilin preparations were found to equal and sometimes even excel the corresponding Feulgen preparations.

Tetrasporangium-initials which arise from the cells of the inner cortex can be recognized by their dense cytoplasm and comparatively large nuclei. The sporangium-initial grows, ultimately reaching its full size, accumulating more and more food, particularly floridean starch. Likewise the nucleus also increases in size and passes through stages which bear a close resemblance to those described by Drew (1934) in *Spermothamnion turneri*. At first the nucleus is near the centre of the sporangium, but during the later prophase stages it lies to one side. In the earliest stages the nucleus has a nuclear membrane and a conspicuous nucleolus, surrounded by a number of small deeply staining bodies or granules. Although these granules appear as distinct entities in both Brazilin and Feulgen preparations, they cannot be counted at this stage as they are numerous and are very close together (Text-fig. 5, A). It has been found impossible to decide whether or not these granules are interconnected, but they are in all probability the heterochromatic regions of chromosomes. In a slightly more advanced stage (Text-fig. 5, B) the granules are definitely connected by less deeply staining threads. Gradually these slender threads take on the appearance of an intricate net-work uniformly distributed within the nucleus (leptotene) (Text-fig. 5, C). In the following stage the threads contract to one side of the nucleus, usually the basal one. Careful observation of the extremities of the threads reveals that they are made up of alternately stained and unstained regions (Text-fig. 5, D and Pl. I, Fig. 2). This appears to be the zygotene stage which, however, is probably of a short duration, the number of sporangia with nuclei at this stage being small.

In the next stage the 'U'- or 'V'-shaped threads are distributed throughout the nuclear area (Text-fig. 5, E and Pl. I, Fig. 3). They appear thicker and more homogeneous than during the preceding stages and this probably represents pachytene. From now onwards until late diplotene the threads do not stain readily and represent the 'diffuse stage' of Westbrook (1928, 1935). The threads become short and straight and lie near the periphery of the nucleus. In favourable Feulgen preparations some of them have the appearance of two threads coiled around each other at one or both ends (Text-fig. 5, G), probably indicating the double nature of the threads. As the nucleus and nucleolus enlarge further, the bivalents become more slender and are studded at intervals with thicker regions. Some of the bivalents are short and straight, while others are long and bent.

The nucleus and nucleolus attain their maximum size in the diplotene stage. The outline of the nucleus is elliptical in section, its long axis being parallel to the long axis of the tetrasporangium. Numerous bivalents can be

seen distributed all over the nuclear area (Text-fig. 5, F). The doubleness of the bivalents manifests itself very clearly as the homologous chromosomes, having presumably become duplicated by now, loose their mutual attraction but are held together by the chiasmata. The bivalents assume various shapes and sizes (Text-fig. 5, H), most of them having only one chiasma and appear rhomboidal or as X's when unterminalized and as rods when terminalized.



TEXT-FIG. 5. A-I. Meiosis in the tetrasporangium. A. Early prophase nucleus showing heterochromatic regions of the chromosomes. B. Darkly staining bodies interconnected by lightly stained regions. C. Slender chromatin threads uniformly distributed throughout the nucleus (leptotene). D. Chromatin contracted towards one side (zygotene). E. U- and V-shaped threads uniformly distributed (pachytene). F. Early diplotene nucleus. Note the large side of the nucleus and nucleolus, the numerous bivalents showing chiasmata and the conspicuous cytoplasmic areas at either pole of the nucleus. G. Twisted chromosomes with forked ends from nucleus shown in Pl. I, Fig. 4. H. Bivalents from a Feulgen preparation. I. Diakinesis. 68 bivalents could be counted. Note the distribution of the bivalents in groups and the cytoplasmic areas at either pole. J. Prophase nucleus of apical cell of a female plant. 65-68 chromosomes could be counted in this nucleus.

(A, B, D, E, and F Brazilin; the rest Feulgen.) $\times 1665$.

Some bivalents have two chiasmata and look like rings, while occasional bivalents have three chiasmata resembling the figure 8. It has often been observed that some of the bivalents are triangular. They are probably bivalents with a single chiasma in which one arm is lying parallel to the line of observation.

During diakinesis the bivalents assume a variety of shapes and sizes. In spite of their great contraction and generally round shape, the individual components of bivalents can sometimes be distinguished, now entirely separate from each other. Counts of the number present, i.e. the haploid number, have ranged from 68-72 (Text-fig. 5, I and Pl. I, Figs. 5 and 6).

The nucleolus stains well with Brazilin up to diplotene or diakinesis, but subsequently it does not appear as homogenous and shining. It becomes opaque and lobed as the prometaphase stage approaches. Ultimately it degenerates and forms a cloudy mass around the bivalents. Neither vacuolation nor disintegration of the nucleolus into separate pieces has been observed.

When all the bivalents ultimately occupy an equatorial position they are crowded together and their individual identity can no longer be made out. This appearance at metaphase has been a very constant feature in all preparations irrespective of the fixatives employed, but should probably not be considered normal. As a result of this, disjunction cannot be observed, but presumably takes place and the two sets of chromosomes move apart as compact plates. The surrounding cytoplasm becomes denser, and as the plates move apart they are surrounded by irregular masses of densely staining cytoplasm.

A wall, the formation of which starts during metaphase, separates the protoplast into two uninucleate cells and thus a diad is formed before the nucleus undergoes the second division. Since such diads are frequently met with, it can be inferred that the interphase between the first and second divisions is one of long duration. Subsequently, each cell divides into two and thus four spores are formed in a tetrasporangium.

Late prophase stages in mitosis have been seen in large numbers in various types of cells such as the apical cells and medullary filaments of sexual and tetrasporic plants, gonimolobes, and spermatangial filaments, but critical counts of chromosomes from these cells could not be obtained due to the large number of chromosomes in relatively small nuclei. A few counts of the chromosomes found have, however, been obtained from the apical cells and medullary filaments of the main axis and apical cells of sterile nemathecial filaments of female plants. Text-fig. 5, J and Pl. VI, Fig. 9 show a prophase nucleus from the apical cell of a female plant in which approximately 65–68 chromosomes have been counted. It has not been possible to obtain accurate chromosome counts of tetrasporic individuals, but the number is sufficiently large to indicate that it is double the haploid number. Pl. I, Fig. 10 shows the nucleus of a medullary filament of a tetrasporic plant in which up to 130 chromosomes have been counted.

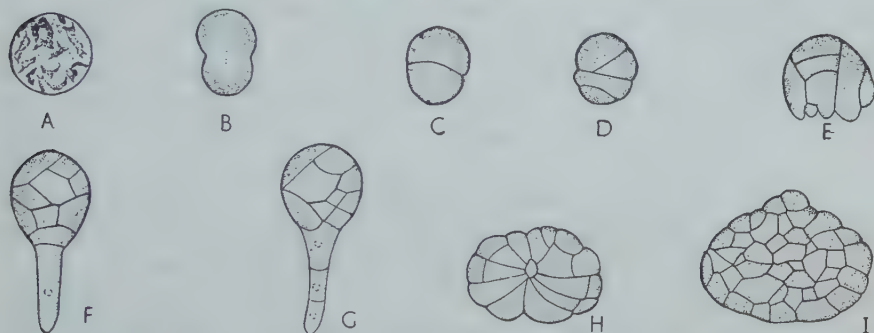
6. EARLY STAGES IN THE GERMINATION OF SPORES

The culture solution and tank used for germinating tetraspores and carpospores and also the culture tank are the same as those employed by Drew (1952). Crystalline penicillin has proved very useful in clearing cultures of germlings of bacteria. A concentration of 10 units of penicillin per 1 c.c. of culture solution (cf. King, 1950) has been used for this purpose.

Tetraspores have been germinated on seven different occasions during the period of this investigation. Sporelings germinated during November 1952 were kept growing for about 13 months, when they were abandoned as growth

became very slow and they were heavily overgrown by diatoms and filamentous algae.

When sections of fertile regions of tetrasporic plants are floated in culture solution contained in petri-dishes the liberated spores, being heavier than water, sink to the bottom. They enlarge and become spherical. The first division in such spores may occur after varying intervals of time ranging from a few days to a month. In one instance, healthy undivided spores could be seen at the bottom of the culture dish 2 months after liberation. The first wall may appear in a spore after it has elongated slightly or without any change of shape. Either one or both the resulting cells participate in subsequent divisions although one of the cells may simply elongate into a septate or aseptate rhizoid (Text-fig. 6, F and G). Subsequent divisions of the spore



TEXT-FIG. 6. Early stages in the germination of tetraspores. $\times 200$.

follow no regular pattern but ultimately a multicellular hemispherical body results. Sometimes several peripheral cells on one side elongate to form rhizoidal outgrowths, which either persist or degenerate again. After a time the rate of growth becomes slow, but small purple-red upright shoots appeared, however, after 4 months on a few of these germlings. Each disk may bear one to three such upright axes. Although a plant was maintained for 13 months, there was no formation of any reproductive organs. The stages in the germination of a carpospore closely resemble those of a tetraspore.

Thuret and Bornet (1878) state that they did not succeed in germinating tetraspores, although they obtained multi-cellular cushions from germinated carpospores. The irregular divisions of the tetraspores and carpospores recall the germination stages of certain Cryptonemiales and Gigartinales figured by Chemin (1937).

7. DISCUSSION¹

This investigation of the development of the reproductive organs, the carposporophyte, and the life-history of *P. caprinus* has revealed features of interest which will now be discussed.

¹ The views expressed here regarding nemathecium and the interpretation of the filaments bearing carpogonia have been arrived at after discussion with Dr. K. M. Drew, who also supports them.

The tetrasporangia of *P. caprinus* develop in unspecialized upper portions of the thallus, endophytes often occupying the cavities left by the dispersal of the spores. In contrast, the sexual reproductive organs develop in special superficial mound-like growths referred to as nemathecium (Kylin, 1923; Taylor, 1937; Fritsch, 1945; Drew, 1951). Long ago, Greville (1824) called them 'spongioles' and Thuret and Bornet (1878) 'plaques'. This restriction of the sexual organs and carposporophyte to such superficial areas—which are later shed—is probably of considerable biological advantage to a perennial species such as *P. caprinus*.

Fritsch (1945) reports that nemathecium occur in the families Rhizophyllidaceae, Squamariaceae, Gigartinaceae, and Phyllophoraceae, but closer consideration shows that the structures in question are certainly not homologous. The term nemathecium appears to have been used by Smith (1938) and Fritsch (1945) to signify any outgrowth of a given appearance irrespective of origin, although Phillips (1925) tries to be more precise than others by restricting its usage to the warty outgrowths bearing tetrasporangia. A clear distinction must be made between structures which arise entirely from the gametophyte (*P. caprinus*) or tetrasporophyte (*Peyssonnelia dubyi*) and contain reproductive organs and those which develop from a generative auxiliary cell and are therefore solely the carposporophyte (*Phyllophora brodiaei*). Among those algae in which the sexual reproductive organs are found in special outgrowths of the gametophyte, it is found that there are two types; firstly, those in which the outgrowth shows the same structure as the main thallus bearing it, as in *Gigartina stellata* (Drew and Greig-Smith, in Newton, 1949), and secondly, those in which the outgrowth is different in structure from the main thallus, e.g. *P. caprinus*. It is to the latter that it would seem best to restrict the use of the term nemathecium, a term first used by C. Agardh (1822) for the wart-like outgrowths found on *Ahnfeltia plicata*. As the significance of these is still doubtful, a fresh definition is much needed and it is suggested that the term *nemathecium* is used for a mound-like growth of specialized filaments produced by the further growth of the superficial cells of the gametophyte or tetrasporophyte and bearing the appropriate reproductive bodies. Where carpogonia occur in the nemathecium, the resultant carposporophytes will in all probability develop inside the nemathecium also.

The carpogonia of *P. caprinus* have been shown to be terminal on certain filaments of the nemathecium. These nemathecium filaments may be interpreted either as fertile branches bearing sessile carpogonia or very long carpogonial branches. The former interpretation has been accepted in this account and the branches in question have been referred to as fertile branches. The reasons for this view are, firstly, the filaments in question are continuations of outer cortical filaments, and secondly, the cells of the filament terminated by a carpogonium are not necessarily any different from neighbouring sterile filaments at the time of fertilization. Many instances have been observed in which the fertilized carpogonium had formed a gonimoblast without any of the subtending cells showing any change of contents. On

the basis of this interpretation of the filament as a fertile branch the carpogonia are sessile. Sessile carpogonia have been reported previously in several members of the Rhodophyta from various orders (e.g. in *Petrocelis hennedyi* and *Cruoria pellita*, Rosenvinge, 1917; in *Rhodochorton violaceum*, Drew, 1935; and in *Gelidium cartilagineum*, Kylin, 1923). In view of these records, it appears likely that sessile carpogonia may be more common in the Rhodophyta than hitherto reported.

There are at present no well-defined criteria for deciding when a carpogonium is mature, but it is generally assumed to coincide with the stage when its trichogyne attains its greatest length. In *P. caprinus* it has been observed that it is the first-formed carpogonia with short trichogynes which are fertilized and that it is likely that carpogonia with very long trichogynes are not. Possibly the trichogynes of unfertilized carpogonia elongate indefinitely, keeping pace with growing sterile nemathecial filaments and ensuring the exposure of the terminal receptive region. In old nemathecium numerous such unfertilized carpogonia with greatly elongated filiform trichogynes are always seen.

There are several features of the organization of this carposporophyte which call for comment. It has been shown that a single branched gonimoblast develops from the carpogonium direct, and that although this fuses with certain cells of the nemathecial filaments, there is no nuclear migration from the gonimoblast to these cells. For this reason and the fact that the carpospore-producing branches, the gonimolobes, develop from the gonimoblast, these cells of the nemathecial filaments are considered to be nutritive auxiliary cells as defined by Drew (1954). It has also been shown that the gonimolobes may arise in various positions and not necessarily in close proximity to the nutritive auxiliary cells. In addition, they outnumber the auxiliary cells. These observations provide additional evidence in support of the views put forward by Drew (1954) that the type of carposporophyte exemplified by *Dudresnaya coccinea*, in which the gonimolobe develops from the auxiliary cell after a transfer of a nucleus from the gonimoblast, has arisen from the type, illustrated by *Sirodotia* or *Dermonema*. In these the spreading gonimoblasts do not fuse with cells of the gametophyte and thus *P. caprinus* is an intermediate type, the gonimoblast fusing with cells of the gametophyte, the nutritive auxiliary cells, but there is no transfer of nuclei and the gonimolobe formation takes place on the gonimoblast, Drew's Group B, in fact.

Evidence has been given for considering the fusions of the gonimoblast with cells of the fertile branch as secondary. They do not appear to be obligatory judging from the small number of examples observed relative to the amount of material examined, and thus differ from those which occur in the Dumontiaceae. It is possible that the fusions are of a completely different category altogether.

The cytological evidence indicates that reduction division takes place in the tetrasporangium of *P. caprinus*. The very high haploid number of *c.* 70 has made accurate counts difficult. There is evidence that the number of

bivalents counted at diakinesis (68–72) roughly corresponds with the haploid chromosome counts obtained from the apical cells of sexual plants. Although counts have not been possible a considerably large number of chromosomes has also been seen in gonimolobes and the medullary cells of tetrasporic plants. It seems reasonable to conclude, therefore, that the life-history of this alga in the British Isles is of the *Polysiphonia*-type, i.e. it is cytologically diphasic (Drew, 1944).

An extended investigation would show whether the same would be true for other localities such as Denmark and Sweden, where the available information, showing that sexual thalli predominate, suggests it might not be. The high numbers of chromosomes, whatever the significance, has no parallel in the investigated Florideae.

In conclusion I wish to express my grateful thanks to Dr. K. M. Drew for her guidance, encouragement, and criticism throughout the course of the investigation and help in the preparation of this paper, and to Professor C. W. Wardlaw for his encouragement and advice. I should like to thank all those who assisted me with the collection of material, especially Dr. M. T. Martin, Dr. S. M. Lodge, and Mr. H. T. Powell. My thanks are also due to Mr. E. Ashby for the photographic illustrations.

I wish to take this opportunity of thanking the authorities of the Banaras Hindu University for enabling me to study in Manchester.

8. SUMMARY

1. Sexual organs develop in superficial nemathecium which arise in a fortuitous manner on the upper dichotomies of male and female plants. Spermatangial filaments are richly branched and brush-like in appearance. Carpogonia are terminal and sessile on undifferentiated fertile branches.

2. Undifferentiated intermediate cells of nemathecium filaments rich in floridean starch and situated at varying distances from the carpogonium, function as nutritive auxiliary cells. They cannot be distinguished until a gonimoblast fuses with them. There is no transfer of the diploid nucleus into the auxiliary cell or any cell of the gametophyte.

3. A single gonimoblast develops direct from the fertilized carpogonium without any prior fusions with the cells of the fertile branch. It branches profusely, fusing with a number of nutritive auxiliary cells and in the early stages bears gonimolobes near the regions of fusion with these cells. During the last stages in its development gonimolobes may be formed on the gonimoblast remote from auxiliary cells.

4. Secondary fusions between the cells of the fertile branch and the base of the gonimoblast have been seen in twelve instances and occur at a late stage in the development of the carposporophyte.

5. Cruciate tetrasporangia develop from cells of the inner cortex of special plants. Reduction division precedes tetraspore-formation. The haploid

chromosome number of 68-72 established from counts at diakinesis is very considerably higher than any previously reported in the Florideae.

Nuclei have been seen in division in apical cells of the thallus, gonimoblast, and spermatangial filaments, but the large number of chromosomes has precluded accurate counts. The cytological evidence suggests, however, that the life-history in the British Isles is of the *Polysiphonia*-type.

6. The germination of tetraspores and carpospores is alike. After some irregular cell-divisions a hemispherical cushion of cells results. Growth of sporelings in culture is very slow, and upright axes arise from the disks only after several months.

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EXPLANATION OF PLATE VI

Illustrating C. S. Prakasa Rao's article on 'The Life-History and Reproduction of *Polyides caprinus* (Gunn.) Papenf.'

Fig. 1. Nucleus of tetrasporangium showing uniform distribution of chromatin. Late leptotene. $\times 2000$.

Fig. 2. Nucleus of tetrasporangium showing contraction of chromatin to one side. Alternate stained and unstained regions visible in the threads. Zygotene. $\times 2000$.

Fig. 3. U- and V-shaped thicker threads showing the stained and unstained regions more clearly. Pachytene. $\times 2000$.

Fig. 4. Straight threads with forked ends. Late pachytene. $\times 2000$.

Fig. 5. Diakinesis. 68 bivalents could be counted in this nucleus. Composite drawing of this nucleus given in Text-fig. 5, I. $\times 2000$.

Fig. 6. Diakinesis. 72 bivalents could be counted in this nucleus. $\times 2000$.

Fig. 7. Part of a transverse section of a very young nemathecium. Note the deeply stained male nucleus within the trichogyne. Very little of base of carpogonium in this section. $\times 1000$.

Fig. 8. Fusion of male and female nuclei within the carpogonium. Note the constricted neck of the trichogyne. The dark areas in the trichogyne are the nuclei of cells behind the trichogyne. $\times 2000$.

Fig. 9. Nucleus of apical cell of female plant. 65-68 chromosomes could be counted in this nucleus. Composite figure of this nucleus shown in Text-fig. 5, J. $\times 2000$.

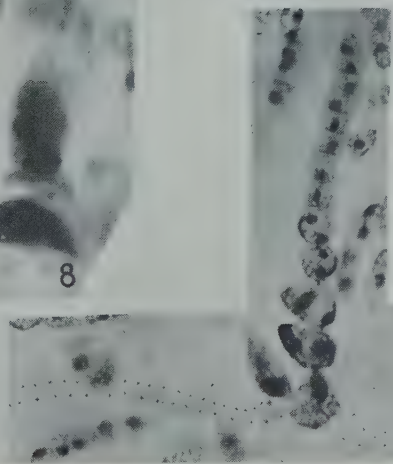
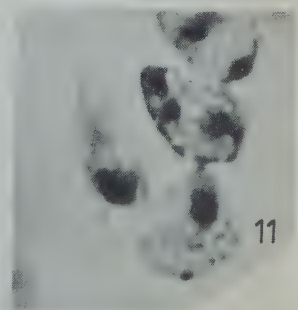
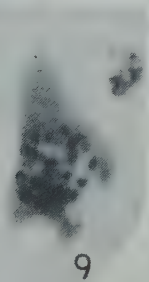
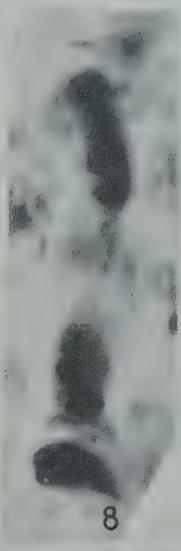
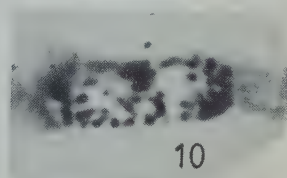
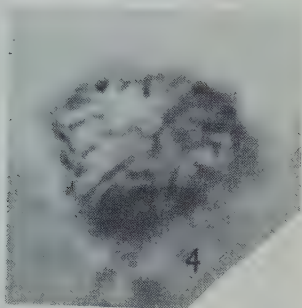
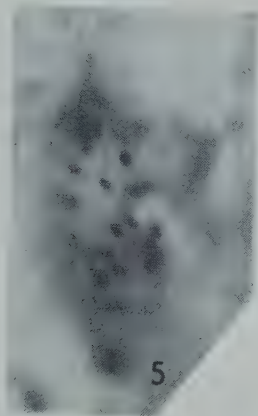
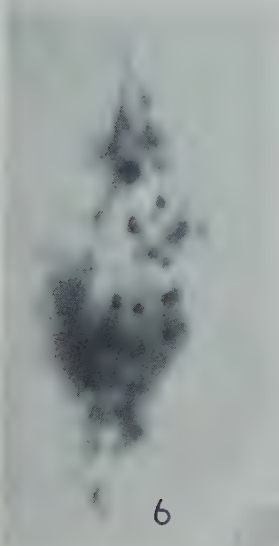
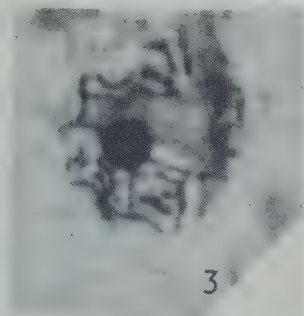
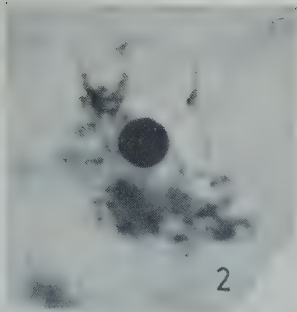
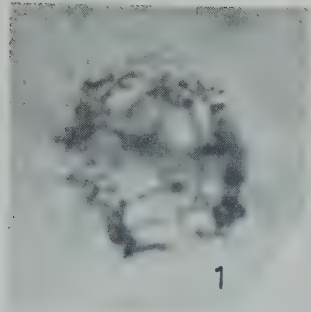
Fig. 10. Nucleus of a medullary filament of a tetrasporic plant. Up to 130 chromosomes could be counted in this nucleus. $\times 2000$.

Fig. 11. Formation of gonimolobe-initial as an outgrowth from the expanded region of contact between the gonimoblast and the nutritive auxiliary cell. Auxiliary cell uninucleate and two cells above multinucleate. $\times 1000$.

Fig. 12. More general view of the auxiliary cell branch of Fig. 11 and the gonimoblast. The latter is very lightly stained and its outline is emphasized with ink. Note the prominent nucleus in its tip. $\times 500$.

Figs. 1, 4-6, 9, 10 Feulgen.

Figs. 2, 3, 7, 8, 11, 12 Brazilin.



Studies in the Regeneration of Horseradish

II. Correlation Phenomena

BY

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With Plate VII and five Figures in the Text

ABSTRACT

Regeneration has been studied in fragments of various sizes, and complete plants, together with the effect of externally-applied β -indolylacetic acid (I.A.A.). The main conclusions are as follows:

(1) In the intact plant regeneration is inhibited by the growing crown; in cuttings, I.A.A. will reduce the rate of primordium-formation, but cannot entirely suppress it.

(2) The bud/root ratio among differentiated primordia is about 0.5–0.9 in long cuttings; it rises sharply in cuttings less than 2 cm. long, and falls in the presence of I.A.A. Most, though probably not all, of the observations concerning this ratio can be explained on a simple auxin-level hypothesis.

(3) I.A.A., at an appropriate concentration, depresses the extension of buds and promotes that of roots. On each cutting, only one (proximal) bud will normally extend—but this form of dominance cannot be exactly simulated by I.A.A., and more than one hormone seems to be involved.

The results are discussed from the point of view of hormone theories of regeneration, and it is concluded that direct measurements of internal auxin-content will be essential before the phenomena can be interpreted with consistency.

I. INTRODUCTION

THE previous paper (Dore, 1955) dealt with the purely descriptive aspects of horseradish regeneration. In any investigation of the accompanying correlation phenomena, the problem to be considered is threefold:

(1) *Initiation*. What factors control the production of new primordia? Roots dug up from plants in the field, small but well past the stage of 'ripeness to regenerate' (Dore, 1955), show no evidence of regeneration; yet such roots, once detached, produce in the laboratory abundant new primordia within 2 to 3 days. Only in old roots does regeneration appear to occur naturally.

(2) *Differentiation*. Since the primordia appear to be at first 'indifferent' in nature, what factors determine whether they shall differentiate into buds or into roots?

(3) *Extension*. Of the many buds differentiated on a length of regenerating root, what factors determine which of these buds shall extend to become new crowns? For by no means all buds do so.

These three aspects are of course inter-related, and any experiment

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designed to investigate one will normally provide information relevant to the others; this paper is therefore necessarily divided according to the type of experiment carried out, rather than according to the process under investigation. The results are presented in an order representing an ascending order of size of regenerating portion, starting with the smallest fragments (excised root-scars) that can regenerate at all, and ending with such few experiments as we have been able to carry out on complete plants.

II. EXCISED SCARS AND PRIMORDIA

Experiment 1—Regeneration in excised root-scars

The smallest valid 'unit' of regenerating material is clearly a plug of tissue including the lateral root-scar and its vascular strand. Plugs 2 mm. long by 1.24 mm. diameter were excised from roots (which had previously been thoroughly washed in soapy water) by means of a sterile exploring needle. This was 2 in. long, of external diameter 2 mm. and internal bore B.W.G. 14, with the normal bevel end ground away to produce what was essentially a micro cork-borer. The plugs were then washed in several changes of sterile glass-distilled water and, on the first occasion, planted on sterile sintered glass disks in petri-dishes containing a mineral solution supplemented by 4 per cent. sucrose (as used by Bonner and Addicott (1938) for the sterile culture of pea roots). Nine scars were so grown, three to a dish, and compared with a set of cortical tissue-plugs exactly similar except that they did not contain a scar or root-trace; the cultures were maintained in darkness at 30° C. for 42 days during which the medium was twice changed in order to reduce fungal contamination. Of the 'control' plugs without scars, all survived the full period, but none showed either growth or regeneration; of the nine excised scars, four died, two survived but remained undifferentiated, one formed a callus, and two produced a single primordium each which did not differentiate further.

On the second occasion, fifteen excised root-scars were used but no controls; the scars were again planted three to a dish, but this time direct on to the surface of culture-medium solidified by the addition of 2 per cent. agar. In order further to reduce contamination, penicillin solution was added to each dish to give a concentration of 10,000 O.U.s per dish. In 7 days, two had died, two remained unregenerated, and the remaining eleven had each produced a primordium, one of which had differentiated into a bud. All the living plugs were grown for a further 40 days, during which there was no further differentiation.

Conclusions 1

The complete absence of regeneration in the absence of the root scar and trace is strongly reminiscent of the observation by Jablonski and Skoog (1954) that vascular tissue promotes cell-division in tissue-culture. The vascular strand of the main root appears to lack these powers; but it must be remem-

bered that the lateral root trace no longer serves its original function, and we may postulate that, when the lateral root dies, chemical changes occur in its remaining vascular strand which culminate in the production of a primordium-stimulating substance. We may also note that the rapid differentiation shown in, for example, 2 cm. cuttings is almost completely suppressed in small tissue-plugs.

Experiment 2—Further differentiation of excised primordia

We hoped that, if undifferentiated primordia were removed from their parent cuttings and cultured, it might be possible to influence the direction of their differentiation by the action of auxin-like compounds. Roots were thoroughly washed in soapy water (calcium hypochlorite and mercuric chloride were both found to be unsuitable), and cut into 2 cm. lengths under aseptic conditions; the cuttings were washed in sterile water, dried on sterilized filter-paper, rolled in flowers of sulphur, and allowed to regenerate on damp sand in petri-dishes, previously autoclaved. After 6 to 7 days primordia were removed with a sterile knife made by flattening and sharpening a needle, washed twice in sterile water, and transferred to the appropriate solution in petri-dishes kept in darkness at 30° C. Bonner and Addicott's medium was again used as a base, substances under investigation (sterilized by passing through a No. 5 sintered glass filter) being added as required. Despite these precautions, some amount of contamination of the cultures was almost invariable, and blank experiments showed conclusively that this contamination was introduced with the primordia. In some of the later experiments penicillin (1,000 O.U.s per ml. culture) was added with advantage.

Results 2 (a)—Effect of β -indolylacetic acid

Primordia were planted three to a dish in culture solution supplemented by concentrations of I.A.A. varying from 10^{-9} to 10^{-3} gm./ml., and examined after 8 days. No roots were formed; the number of buds, and the number of undifferentiated primordia still remaining, are shown in Table I. It will be

TABLE I
Effect of I.A.A. on Differentiation of Excised Primordia

Concentration of I.A.A.	0	10^{-9}	10^{-8}	10^{-7}	10^{-6}	10^{-5}	10^{-3}
Undifferentiated	14	9	9	11	11	10	10
Buds	4	4	3	1	1	2	2

observed that, even at the high concentration of 1 gm./litre, I.A.A. has had little effect; there is, though, a suggestion that in the higher concentrations (10^{-7} and above) production of buds is depressed.

Results 2 (b)—Effect of other substances

Changes in the concentration of α -naphthylacetic acid and adenine (over the ranges investigated) had even less effect than in the case of I.A.A. We

have therefore taken the unusual course of combining the results for *all* concentrations of each substance for comparison with the controls, and they are presented in this form in Table II; the detailed results may be consulted in the thesis (Dore, 1954). The term 'root+bud' refers to those cases in which the primordium divided—one-half, as is usual in such cases, becoming a root, the other a bud. The results for I.A.A. have been included for comparison.

TABLE II

Effect of I.A.A., N.A.A. and Adenine on Differentiation of Excised Primordia

Period of growth	Conditions	Numbers of primordia				Percentage of primordia			
		U	R	B	R/B	U	R	B	R/B
8 days	Control	14	—	4	—	78	—	22	—
	I.A.A.	60	—	12	—	83	—	17	—
10-13 days	Control	16	4	15	1	44	11	42	3
	N.A.A.	34	13	55	4	32	12	52	4
11 days	Control	20	4	28	1	38	7	53	2
	adenine	20	8	24	2	37	15	44	4

U = undifferentiated; R = root; B = bud; R/B = root+bud.

The most striking feature of these results is the low proportion (ultimately about 59 per cent.) of primordia which differentiates at all. This is not simply a time factor; although excised primordia have been kept alive (and apparently healthy) for 30 days, no further differentiation has been observed in any experiment after 11 days. A further feature is the low ratio of roots to buds produced. This is a characteristic feature of regeneration from small portions of root (*vide* Section IV) and may well be related to the fact (Dore, 1955) that buds differentiate more rapidly than roots.

The growth-substances have had little, if any, effect. There is a suggestion that N.A.A. favours the production of buds, and adenine (in contrast to the findings of Skoog and Tsui (1951)) that of roots; but in view of the erratic behaviour of the controls, little reliance can be placed on this observation.

Results 2 (c)—Other effects

First, the experiments summarized in Table II included some in which aneurin and nicotinic acid were added to the culture solution, but without effect. Secondly, in most of the experiments the excised primordia were classified according to whether they had been removed from the proximal or distal halves of a cutting. The results were largely inconclusive, though in such few cases as any distinction was apparent there was a tendency for proximal primordia to differentiate into buds and distal primordia into roots. This is in keeping with the weak polarity shown by complete cuttings (Section V).

Conclusions 2

The failure of many primordia to differentiate is probably no more than a 'size effect' similar to that reported by Brown and Wightman (1952) for isolated

pea roots and by many workers for immature angiosperm embryos. Cell-division, development, and differentiation seem to depend on a supply of metabolites that meristems alone cannot provide, so that these processes are markedly dependent on the amount of mature tissue present. With almost 60 per cent. of primordia remaining undifferentiated, it is impossible to draw any conclusions as to the nature of the effects of external auxins; the small effects noted, even if real, are as likely to be due to the selection of primordia already determined as to any direct influence on the direction of determination. In fact, the slight polarity shown suggests that by the time that primordia are large enough to differentiate after excision their future (i.e. bud or root) is already settled. Even the high bud/root ratio may mean no more than that buds, which on cuttings develop more rapidly than do roots, are more self-sufficient in their metabolic requirements; so that primordia already determined as buds continue to differentiate, whereas the majority of those already determined as roots do not. It is now clear that, in the absence of a culture technique which can supply all that is normally supplied by mature tissue, the further study of excised primordia is unlikely to be illuminating.

III. THIN ROOT SLICES

Experiment 3—Regeneration in root-slices

Roots not more than 5 mm. diameter from plants 6 months old were washed in soapy water and then in water; from these were cut 160 1-mm. slices (duplicated experiments, twenty slices in each of four treatments). As the slices were not specially selected, some were without root-scars. They were planted on 16 ml. of inorganic medium mixed with enough sand to provide a solid substrate and with added I.A.A. to give concentrations of 10^{-3} , 10^{-4} , 10^{-5} , and zero. The experiments were carried out at 30° C. and examined daily for 25 days; I.A.A. was renewed twice in the course of the experiment.

Results 3 (a)—Time of onset of regeneration

The number of days (to the nearest half-day) from the beginning of the experiment to the first appearance of regenerated structures is shown in Table III. The behaviour of the controls is substantially as expected except

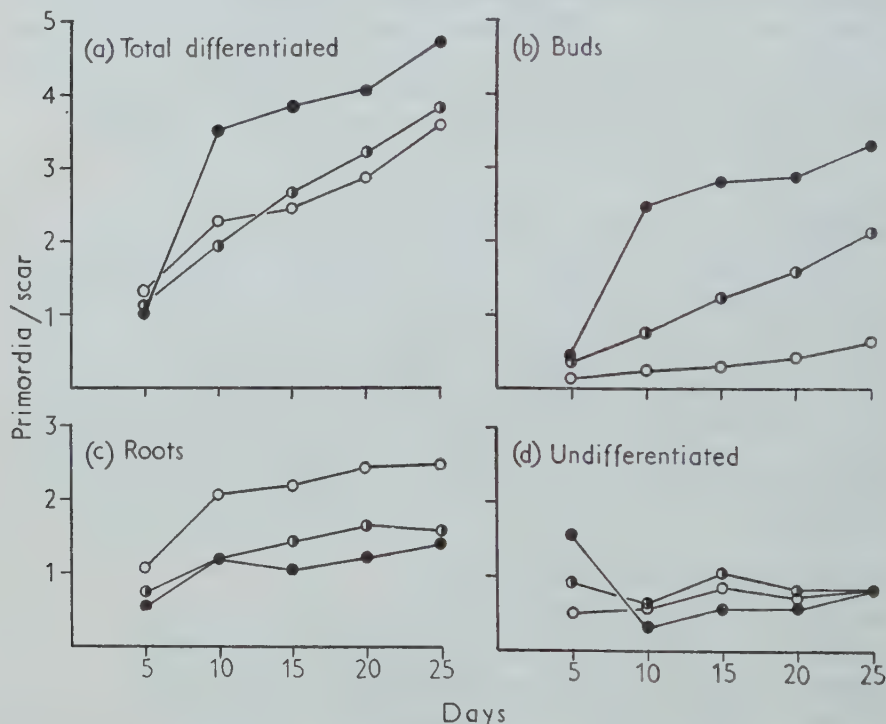
TABLE III
Effect of I.A.A. on Regeneration in 1 mm. Slices
(Time in days of first appearance)

	Primordia	Roots	Long roots (> 1 mm.)	Buds	Leafy buds
Control	3	5.5	6.5	5	12
10^{-5}	2.5	4	5.5	5	17.5
10^{-4}	2	4	4.5	4.5	Do not elongate
10^{-3}	2.5	4.5	6	Do not appear	Do not appear

that roots are recognizable as such somewhat earlier than is usual in complete cuttings. There is a suggestion of a slight hastening in the appearance of both primordia and roots as a result of the auxin treatments; but in view of the known variability of horseradish in this respect, no reliance is placed on this result. There is, though, a striking effect on buds: a concentration of 10^{-4} suppresses their extension, and 10^{-3} inhibits their appearance completely.

Results 3 (b)—Root-bud relationships

A concentration of 10^{-3} I.A.A. was found to be toxic, all slices dying after 15 days; results for this concentration have therefore been excluded from the account which follows. The time-course of regeneration, expressed in primordia per scar, is shown in Text-fig. 1 for (a) total differentiated primordia, (b) buds, (c) roots, and (d) undifferentiated primordia. It is noteworthy



TEXT-FIG. 1. Effect of I.A.A. on regeneration in thin slices. Closed circles, controls; half-closed circles, 10^{-5} I.A.A.; open circles, 10^{-4} I.A.A.

that the extent of regeneration is very high, attaining in the controls nearly 5 primordia/scar; in complete cuttings a value between 1 and 2 is more usual.

Text-fig. 1 (a) shows that there is a great increase in total differentiated primordia between 5 and 10 days; this is also a period of active differentiation, as is shown by the corresponding drop in the number of undifferentiated primordia. The rate of primordium-formation falls abruptly after 10 days;

and, since it is about this time that extension-growth of buds begins, it is tempting to suggest that some substance produced by the extending buds depresses, though does not completely inhibit, the initiation of new primordia. Since the lower rate of primordium-formation is maintained from the start in the presence of I.A.A., it seems possible that the fall in initiation is itself brought about by auxin produced by the extending buds. Undifferentiated primordia, apart from the early fall in the controls, remain at an approximately constant level under all conditions throughout the experiment; but differentiated buds (Text-fig. 1 (b)) and roots (Text-fig. 1 (c)) show striking auxin effects. Bud numbers are depressed, root numbers promoted, by I.A.A.; in the case of the two I.A.A. concentrations, with approximately equal numbers of total differentiated primordia, the proportion of buds in both concentrations tends to rise with time; but this is a rise from 33 to 56 per cent. in the 10^{-5} solution and a rise from 9 to 20 per cent. in the 10^{-4} solution.

Results 3 (c)—True adventitious roots

On the slices in 10^{-4} and 10^{-5} I.A.A. a few roots arose from the cortex not in association with a scar. These, which always appeared between 10 and 15 days after the beginning of the experiment, represent the only cases we have seen in horseradish of roots so arising.

Conclusions 3

It is clear that the horseradish can be added to the several examples of regeneration in which it is already established that a high auxin-level promotes the production of roots but depresses that of buds. The bud/root ratios are themselves instructive. At 15 days the controls show a value for this ratio of 2.8—lower than that for excised primordia (in which the mean of all observations gave a value of 5.6)—but still higher than that for a more complete cutting. At an I.A.A. concentration of 10^{-5} the ratio falls to the 'normal' value of 0.9; and it seems possible that ratios of this order, so characteristic of cuttings of 2 cm. and over in length (*vide* next section), are due to the existence of a steady low internal auxin concentration. At a concentration of 10^{-4} the ratio falls still farther to 0.13, a value never occurring under natural conditions; and at 10^{-3} it falls finally to zero, since only roots are produced.

However, though auxin may bear much of the responsibility for bud-root balance once initiation of new primordia has begun, the experiments suggest that auxin alone cannot prevent, though it can reduce, the production of new primordia; even at a concentration that proved rapidly toxic, primordia arose at the normal time. It could of course be argued that this is simply a time-effect: the auxin penetrating so slowly that initiation begins before an internal inhibiting level can be attained. Only work on internal auxin levels can settle this question; meanwhile, it would be wise to bear in mind the possibility that a distinct primordium-inhibitor exists in the intact root.

IV. SHORT CUTTINGS

Experiment 4—The effect of cutting length

The high bud/root ratios obtained with excised primordia and thin slices are also seen in Lindner's (1940) results for tissue-plugs; and it is clearly desirable to ascertain at what size of cutting the ratio drops to its 'normal' value of just below unity. Roots of not more than 5 mm. thick were prepared in the usual way and cuttings taken from them of 1, 2, 5, and 10 mm. in length; buds and roots were counted after 25 days and the bud/root ratio calculated. The mean values for the length of the longest root and bud on each cutting were also noted.

Results 4 (a)—Bud/root ratio

Bud/root ratio is plotted against cutting length in Text-fig. 2, in which have also been inserted (i) the value for excised primordia (5.6) as representing cutting-length zero, and (ii) values for longer cutting-lengths extracted from the results of the next section. For lengths down to 2 cm. the value remains substantially constant at just below unity; but as cutting length is further reduced the bud/root ratio steadily rises. (An abnormally high value (10.5) at 2 mm. seems likely to be accidental and, pending further observations, we have ignored it.) This result can clearly be accommodated within the simple auxin hypothesis (Conclusion 3 above): the smaller the cutting, the more of the internal auxin will be lost, either by diffusion into the medium on which the cutting rests, or possibly even by enzymic oxidation at the cut surface; and the lower the auxin-content, the higher the ratio. The hypothesis is not, however, so all-embracing as to justify complacency. We have briefly investigated the effect of lengthy (10 days) immersion in water on 2-cm. cuttings; regeneration is almost completely suppressed under these conditions, but when the cuttings were transferred to damp sand they produced an abnormally *low* bud/root ratio. If auxin had been diffusing out into the water an unusually high ratio would have been expected.

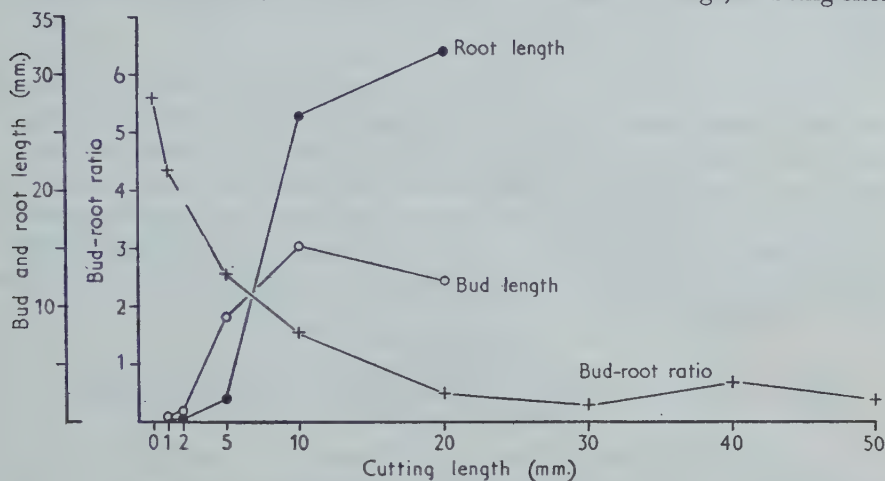
Results 4 (b)—Bud and root length

The values are included in Text-fig. 2. As cutting length is increased, the size of buds rises before that of roots; this can certainly not be ascribed to an increase in auxin, since there is abundant evidence throughout this investigation that auxin *depresses* extension-growth in buds. The apparent interdependence of root and shoot growth in cuttings and seedlings has of course long been familiar, and has led Went, Bouillenne, and others to postulate a series of accessory hormones, the calines (*vide*, for example, Audus, 1953, for references and discussion); our results merely provide yet another example from this group of phenomena.

Experiment 5—The effect of regenerated buds on further regeneration

The experiments with slices (*vide* Results 3 (b)) suggested that the differentiating buds produced a substance which depressed further initiation. An

attempt was now made to ascertain whether this hypothetical substance was likely to be auxin, or whether the postulation of a separate primordium-inhibitor was necessary. It was decided to use 2-cm. cuttings, as being short



TEXT-FIG. 2. Effect of cutting length on bud/root ratio and extension. Crosses: bud/root ratio. The value at 2 mm. has been omitted (see text). Values for 6–20 cm. are not shown; they vary irregularly between 0.45 and 1.15. Circles: bud and root length.

TABLE IV

Effect of Removal of Buds on Root Length (4 cuttings per treatment)

Figures represent mean root length per cutting in mm.

	Treatment		
	A	B	C
	11.00	10.60	18.88
	20.29	5.33	11.00
	14.33	8.44	22.00
	10.43	8.00	16.86
Mean:	14.01	8.09	17.18

A = controls; B = buds removed; C = buds removed and I.A.A. added.

For details see text (Expt. 5 and Results 5 (a)).

enough to handle conveniently in Petri-dishes yet long enough to give normal bud/root ratios. Such cuttings were therefore grown for 14 days to allow leafy buds to develop; they were then subjected to one of three treatments:

A. Controls—no treatment.

B. All leafy buds (i.e. those over 5 mm.) removed, and lanoline-and-water paste applied to the scars.

C. As B, but paste contained 1 in 10^{-3} I.A.A.

Five cuttings were used for each treatment, and the experiment repeated on three occasions; primordia were counted daily, but only the final results (at

the end of 10 days) need be quoted. The total length of roots produced by each of 12 cuttings (4 from each treatment) was also measured, and we shall consider these results first.

Results 5 (a)—Extension growth of roots

The results are given in Table IV, as mean length per root. Analysis of variance of these results, followed by 't' tests between the treatment means, shows that the significant treatment difference is between B on the one hand, and A and C on the other. In other words, the removal of buds—and of the auxin-supply that they, as young apices, are presumably providing—depresses root extension growth; but the application of auxin externally restores growth to its former level. There is, then, no difficulty in explaining these results on an auxin hypothesis.

TABLE V

Effect of Bud Removal (alone or with applied I.A.A.) on initiation of Further Primordia

(Each figure represents mean primordia per scar for 5 cuttings)

		Treatment		
		A	B	C
Roots	I	0.459	0.117	0.249
	II	0.616	0.211	0.418
	III	0.811	0.327	0.332
	Mean	0.629	0.218	0.333
Buds	I	0.531	0.558	0.361
	II	0.436	0.648	0.418
	III	0.449	0.649	0.423
	Mean	0.466	0.618	0.361
Total	I	0.972	0.674	0.665
	II	1.027	0.793	0.701
	III	1.250	0.976	0.729
	Mean	1.083	0.814	0.698

A = controls; B = buds removed; C = buds removed and I.A.A. added.

I, II, III represent separate occasions.

For details see text (Expt. 5 and Results 5 (b)).

Results 5 (b)—Analysis of primordium numbers

The results (as mean primordia per scar) are given in Table V. If we neglect for a moment the changes in *total* primordia, the changes in bud/root ratio can easily be explained; when leafy buds are removed, auxin supplies are reduced and the ratio rises; when auxin is supplied externally, the ratio falls again to normal. However, the reduction in total primordia when the buds are removed, and the further reduction when auxin paste is added, cannot we believe be accommodated within the simple auxin-level theory. Nor does the postulation of a separate primordium-inhibitor assist; and this phenomenon, which we hope to investigate further, remains recalcitrant to our theories.

Conclusions 4 and 5

The behaviour of short cuttings serves only to reinforce the conclusions reached as to regeneration in slices: that the course of differentiation of the primordia—i.e. into buds or roots—can be explained, though not perhaps completely, on a simple auxin-level hypothesis; but that the *initiation* of primordia cannot be so simply explained.

V. CUTTINGS ABOVE 2 CM. LONG

Experiment 6—Effects of cutting length and applied auxin

Cuttings varying in length from 2 cm. to 14 cm. were treated as follows:

- A. Lanoline-water paste applied to proximal end.
- B. Lanoline-auxin paste applied to proximal end.
- C. Lanoline-auxin paste applied to distal end.
- D. Lanoline-auxin paste applied to both ends.

A and *B* were regarded as control and main treatment respectively, and were replicated where possible (from 1 to 6 replicates); *C* and *D* were unreplicated. Results for all four treatments were obtained for cutting lengths from 3 cm. to 10 cm. inclusive; results for 2, 12, and 14 cm. were obtained for *A* and *B* only. In the analyses of covariance which follow, and in the calculation of regression lines, values for these three lengths have therefore been excluded; but the appropriate points have been included in the graphs, and are in good agreement with the regression lines calculated without them. Results were recorded at the end of 25 days' growth on damp sand in enamel dishes covered with black cloth at room temperature; they will be considered under six headings.

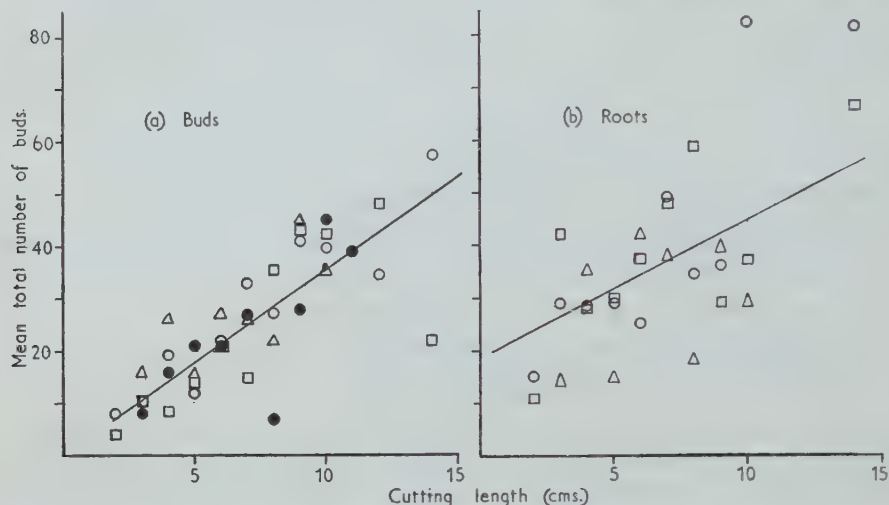
Results 6 (a)—Mean total number of buds

Text-fig. 3 (*a*) represents the relationship between mean total buds and length of cutting, all treatments being included in the same graph. The analysis of covariance is given in Table VI, from which it will be clear that only the joint regression line is significant. This ($y = 3.47x + 0.64$) has been drawn on the graph.

Since the scars of lateral roots are fairly evenly scattered along the regenerating root, it is not surprising that there is a substantially linear relationship between number of buds and cutting length. For lengths less than 2 cm. we have already seen that this relationship breaks down strikingly; and we have reason to believe that for lengths of 20 cm. and above the polarity effects already discernible at the lengths under discussion (*vide* Results 6 (*c*) below) increase to an extent to which this simple relationship once more fails. It is evident that auxin, which (from effects described below) appears to have penetrated the root, has had in this case no effect on the number of buds regenerated.

Results 6 (b)—Mean total number of roots

Text-fig. 3 (b) represents the relationship between mean total roots and length of cutting, all three treatments being included in the same graph (no



TEXT-FIG. 3. Effect of cutting length and I.A.A. treatments on total number of buds and roots. Open circles, treatment A; squares, B; triangles, C; closed circles, D.

TABLE VI
Analyses of Covariance of Total Bud and Root Numbers
(Experiment 6)

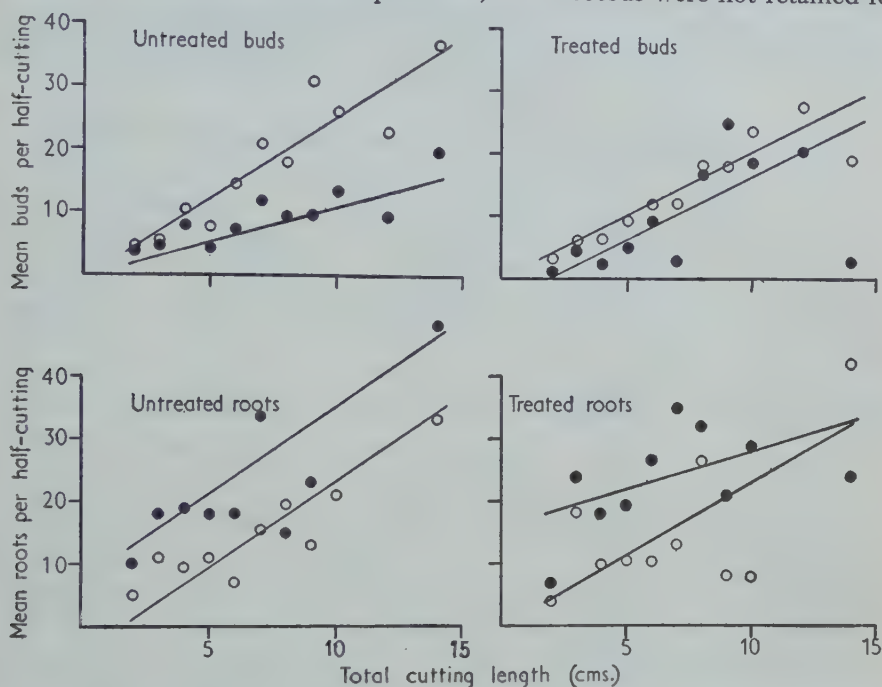
Source of variation	Buds			Roots		
	Squares	D.F.	Mean square	Squares	D.F.	Mean square
Between treatment means	154.37	3	51.46	550.90	2	275.45
Within treatments	4186.82	28	149.52	4191.34	21	199.58
Joint regression	2027.66	1	2027.66***	826.73	1	826.73*
Deviations from joint regression	2159.16	27	79.97	3364.61	20	168.23
Between treatment regressions	234.09	3	78.03	500.18	2	250.09
Residue	1925.07	24	80.21	2864.43	18	159.14

counts were obtained for treatment D). The analysis of covariance has been included in Table VI, from which it will be clear that once more only the joint regression line is significant, and that somewhat precariously; this line ($y = 2.56x + 18.88$) has been drawn in. Again, auxin has had no effect on the total number of roots regenerated.

Results 6 (c)—Distribution of buds and roots on untreated cuttings

For this part of the investigation records are available only for conditions A and B; 'treated' cuttings are therefore those to which an auxin paste was

applied at the proximal end. The cuttings, whose total bud and root numbers we have already considered, were divided into proximal and distal halves and the buds and roots recounted. Two points require elucidation. First, in the graphs (Text-fig. 4) proximal and distal organs for each cutting length are plotted against the *total* length of the cutting. Secondly, owing frankly to our indecision at the start of the experiment, such records were not retained for



TEXT-FIG. 4. Effect of proximally applied I.A.A. on bud and root distribution. Open circles, proximal half; closed circles, distal half.

all replicates. In this section, statistical analyses were carried out on cutting means; in the next, on all replicates separately available. This is the explanation of the wide variation in the number of degrees of freedom shown in the analyses of covariance given in Table VII. Considering first the buds, we have for the regression lines:

$$\text{proximal } y = 2.60x - 0.74$$

$$\text{distal } y = 1.13x - 0.32.$$

These two lines are of significantly different slope, and are separately significant. Distribution of buds, therefore, discloses a definite polarity: there are rather more than twice as many buds on the proximal as compared with the distal half of the cutting.

The root regression lines are:

$$\text{proximal } y = 2.73x - 4.01$$

$$\text{distal } y = 2.73x + 7.78.$$

These lines differ significantly in intercept but not in slope. There thus appears to be a distal formation (probably initial) of about twelve roots irrespective of cutting length; after this, roots are produced evenly along the cutting.

Results 6 (d)—Distribution of buds and roots on treated cuttings

The results in this experiment showed much greater variability; for this reason statistical analyses were carried out on the separate replicates. In order

TABLE VII
Analyses of Covariance of Proximal (P.) and Distal (D.), Bud and Root, Numbers (Experiment 6)

Source of variation	Untreated				Treated			
	Buds		Roots		Buds		Roots	
	D.F.	Mean Square	D.F.	Mean Square	D.F.	Mean Square	D.F.	Mean Square
Between P. and D. means .	1	410.23	1	696.20**	1	300.59*	1	549.14
Within P. and D.	20	68.00	18	166.65	80	93.98	26	81.18
Joint regression .	1	992.79	1	1751.31***	1	3387.63***	1	95.17
Deviations from joint regression .	19	19.33	17	73.44	79	52.29	25	80.62
Between P. and D. regressions .	1	153.17**	1	105.59	1	24.01	1	353.40*
Residue .	18	11.89	16	71.43	78	52.65	24	69.25

not to complicate the graphs, however, only cutting means are shown, and it is for this reason that the lines do not run their expected course through the points, which are not equally weighted.

For buds we now have the regression lines:

$$\begin{aligned}\text{proximal } y &= 2.03x - 0.40 \\ \text{distal } y &= 2.03x - 4.00.\end{aligned}$$

These two lines differ significantly in intercept but not in slope; the intercept difference, amounting on average to only four buds, is perhaps comparable to the group of *c.* 12 distal roots on untreated cuttings. The effect of auxin, therefore, has been to even out the distribution of buds; but since the total is unchanged (Results 6 (a) above), this must mean that there are now fewer buds at the proximal end, and more at the distal end.

For roots we have the lines:

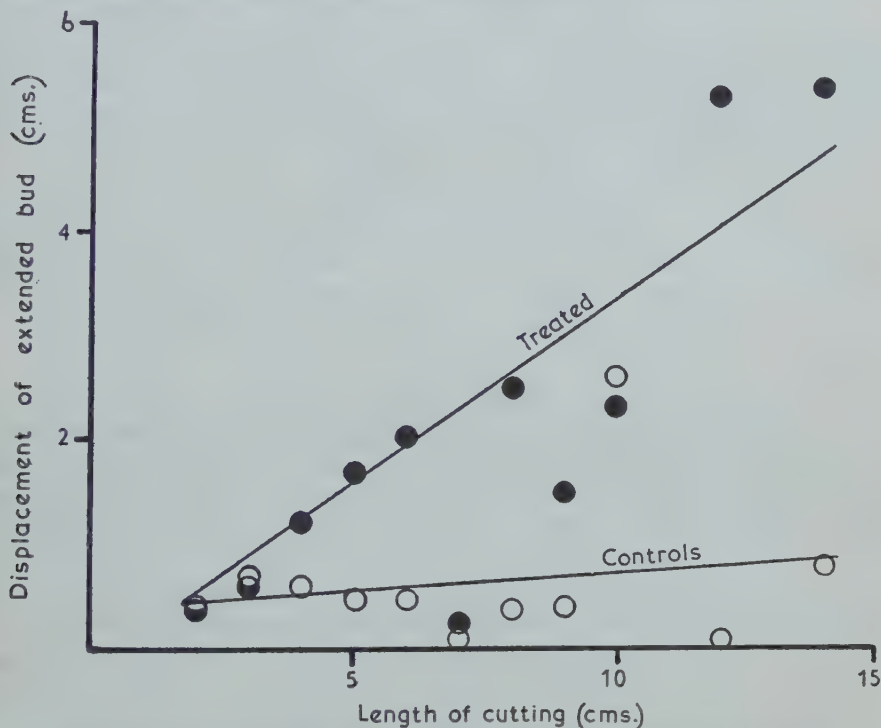
$$\begin{aligned}\text{proximal } y &= 2.31x - 0.16 \\ \text{distal } y &= 1.21x + 15.84.\end{aligned}$$

These lines differ significantly in slope. Inspection of the graph shows even more clearly than before the 'initial advantage' of the distal end; but, after

the initial 12 or so roots have been produced, distribution of subsequent roots is no longer even, there being more roots formed towards the *proximal* end. The significance of these results is considered below.

Results 6 (e)—Position of elongating bud

It has already been observed (Dore, 1955) that normally only one bud on a cutting will elongate to form a new crown; and preliminary experiments on intact plants had already suggested (a) that this is a bud very near to the



TEXT-FIG. 5. Effect of proximally applied I.A.A. on position of extended bud.

proximal end of the detached root, and (b) that the application of auxin to the proximal end inhibits the buds in its immediate vicinity; so that the bud which elongates is now some distance from the proximal end. The left-hand cuttings shown in Pl. VII (i) are a set of Group A (untreated) cuttings; and it is clear that, irrespective of length of cutting, the only bud that has elongated is very close to the proximal end. The distance of this bud from the end is plotted against length of cutting in Text-fig. 5 (open circles) and the regression line ($y = 0.038x + 0.351$) drawn in. The slope of this line is not significant, and the average distance of the bud from the end is less than a half-centimetre. The cuttings (group B) treated with auxin at the proximal end are shown on the right-hand side of Pl. VII (i), and the corresponding graph of distance from proximal end against cutting length has been included in Text-fig. 5. Not

only is there a marked displacement of the elongating bud; this bud is not, as might be expected if a simple auxin-concentration effect were concerned, at a fixed *distance* from the treated end; it appears to be displaced further in the longer cuttings, so as to be set at a constant *proportion* of the total length from the end. This is supported by Text-fig. 5. Only means have been entered on the graph, but the regression line has been calculated from all available replicates; it is ($y = 0.353x - 0.179$), and the slope is significant at the ($P = 0.001$) level. Although there is great variation (very clear from the single set of cuttings shown in Pl. VII (i)), there is a striking tendency for a bud about one-third of the distance from the proximal end to elongate. The unreplicated treatments *C* and *D* were less conclusive but they suggested that the application of auxin to the distal end of the cutting is without effect. Transport of auxin along the root-cutting is thus presumably markedly polar.

Results 6 (f)—Extension growth in buds and roots

In groups *A* and *B*, the lengths of the elongated bud, and of the longest root, were measured. The detailed results are given in the thesis, and it will suffice here to note that auxin not only displaced the elongating bud, but significantly reduced its length; it was, however, without effect on roots.

Conclusions 6

The 'displacement' phenomenon (Results 6 (e)) is, so far as we know, novel. It shows that I.A.A. has its usual effect of suppressing bud extension, and it may well be that polar transport of auxin in the detached cutting results in a deficiency of auxin at the proximal end, so producing a high proximal bud/root ratio (Results 6 (c)) and favouring the early extension of a proximal bud. This bud, by producing auxin in its turn, would then inhibit the remainder. But when auxin (I.A.A.) is *externally* applied, no simple auxin-diffusion theory can account for the fact that the first extending bud is set at a given *proportion* of the cutting-length from the proximal end. On a bio-electric hypothesis—the cutting being regarded as a potentiometer—the phenomenon would present no difficulty; but if we are to maintain a hormone theory, then *two* diffusion gradients are needed. If we postulate (*a*) that separate substances diffuse linearly from each end, starting at comparable levels but travelling at different rates, and (*b*) that a bud extends at the point where the concentrations of the two substances become equal, then the observed phenomenon will follow; though implausible, this at least suggests that a single-hormone theory is inadequate.

The lack of effect of I.A.A. on total primordium numbers is only to be expected; the experiments with slices (*vide* Conclusions 3 above) have already provided an essentially similar result. The bud/root ratio at the proximal end has been reduced in the expected manner; the compensating rise at the distal end is, however, again difficult to explain plausibly on a single-hormone hypothesis. All in all, it seems that when fragments or slices are allowed to regenerate in an artificial environment, the environment so dominates the process that, if only one hormone is applied, only one hormone is needed to

explain the results. As we progress to longer and still longer cuttings, so the phenomena prove increasingly difficult to fit into any such simple hypothesis.

VI. COMPLETE PLANTS

Experiment 7—Severance of tap-root

Limitations of space and material of sufficient age prohibited all but small-scale exploratory observations. Four 8-month plants, grown from regenerated buds in sand in 6 in. plant-pots, were treated as follows:

A. Control—untreated.

B, C. Crown separated by a cut from roots; this necessarily also subdivided the isolated root system into several separate parts.

D. As B and C, but both cut surfaces treated with auxin paste (equal parts anhydrous lanolin and 10^{-3} aqueous I.A.A.).

All plants were examined one month after treatment.

Results 7 (a)—Main experiment

The control plant showed only very few regenerated buds, and those at the base of the plant—i.e. on the region farthest from the crown yet capable of regeneration. In all three remaining plants a small number of buds had formed at the *proximal* end of each portion of the root system; no buds were formed above the cut. New roots were never found below the cut, but in the auxin-treated plant abundant new roots were formed above it. These, however, were largely, if not entirely, formed in stem-tissue and will not be further considered. In B and C, one bud at the extreme proximal end of each part of the root-system had grown considerably; in D, the extended bud was displaced some few inches from the tip (cf. Results 6 (e)).

Similar experiments carried out on four plants *in situ* in the field were less conclusive, for the plants were much older and already showed a number of 'tubercles' though no active regeneration. Nevertheless, there was again a noticeable increase in regeneration at the proximal end of the root system after it had been isolated from the crown.

Results 7 (b)—Parallel observations

Automatic regeneration with age was well shown by two plants, each about 8 months old, whose tap-roots had emerged from the base of the plant-pot. In each case a new crown, together with 2 or 3 small buds, had formed about 20 cm. away from the parent crown; one of these plants is shown in Pl. VII (ii), and the curious 'borrowing' of the existing tap-root by the new crown should be noted. In eight further potted plants, however, a new phenomenon was observed; the main tap-root of these plants regenerated far more vigorously than in any case seen by us in the field, producing primordia in conspicuous longitudinal rows, marking the sites of the old root-scars which in turn (Beijerinck, 1887) follow the protoxylem traces (Pl. VII (iii)). None of these plants had had completely normal growth conditions; in four the tap-roots

had been severed where they inconveniently emerged from the pot, and the remainder had been grown throughout the winter under somewhat inadequate artificial light.

Conclusions 7

(a) These results suggest that *initiation* of new primordia is inhibited by the crown; regeneration will thus only occur at some considerable distance (20 cm. in the cases observed) from an existing crown, and crowding of new shoots is avoided. If we postulate a primordium-inhibiting hormone, this apparently cannot be auxin; the extension phenomena suggest that the I.A.A. had been taken up by the root, but regeneration was not thereby prevented. The eight abnormal cases of regeneration close to the crown (which incidentally also serve to show that the oxygen supply necessary for regeneration (Dore, 1954) was adequate) were associated with unsatisfactory growth conditions; and it may be that the primordium-inhibitor is produced only by an actively growing crown.

(b) The fact that no new roots were observed below the cuts suggests that the presence of existing root-apices inhibits the *differentiation* of primordia into roots.

VII. REVIEW OF PROBLEM

We may now return to the threefold problem posed in the Introduction and summarize our (partial) answers to the questions there raised:

1. *Initiation*. In the intact plant the crown inhibits regeneration in the root, so that regeneration will occur only in a region either sufficiently far removed from the crown, or artificially separated from it. The phenomenon cannot be reproduced by the application of I.A.A., and it seems certain that, if there is a specific primordium-inhibitor, it is not I.A.A.

2. *Differentiation*. This has been the main object of our investigations. Most—though perhaps not all—of the phenomena concerned can be explained on a simple auxin-level hypothesis: high auxin levels favour roots, low levels favour buds.

3. *Extension*. Of the phenomena concerning the extension of differentiated organs, most—though certainly not all—can be explained on a simple auxin-level hypothesis: auxin depresses the extension of buds, but promotes that of roots.

Nevertheless, to translate even these simplified conclusions into a precise statement of the postulated changes in auxin content and distribution when a root is detached would be difficult and probably unrewarding; and it must always be remembered that it is the phenomena which *cannot* be simply explained which are likely to prove of the greatest interest. We have throughout tacitly assumed a hormone theory of regeneration, as being the type of theory most accessible to verification. In setting up such a theory, however, one of two policies can be adopted. First, we may postulate as many hormones as are necessary to explain the phenomena completely; this has been done

by one of us (J.D.) in the thesis (Dore, 1954) from which this paper has been taken, and it was found that not less than five hormones are needed. Secondly, we may explain as many of the phenomena as possible by postulating only one hormone (auxin) at varying levels. The first provides the clearer picture of the complexity of the process under investigation, and is likely—ultimately—to be the more accurate; the second distinguishes more clearly the unfamiliar from the familiar properties, and serves to attract attention to those aspects likely to be most fruitful for further study. It is for this reason that we have chosen the second course in preparing this account.

It is hoped to carry the work farther at Southampton and to make direct estimations of the internal auxin levels. In much auxin work the obvious explanation is perhaps at times too readily accepted; in the familiar case of apical dominance, for example, it is often argued that because the inhibiting effect of the apex can be simulated by the application of auxin, then auxin is responsible for the apical effect in the intact plant. This may be true; but as an argument it may be summarized in the form [If P , then Q ; but, Q ; therefore P], and is known to logicians as the Fallacy of the Affirmation of the Consequent.

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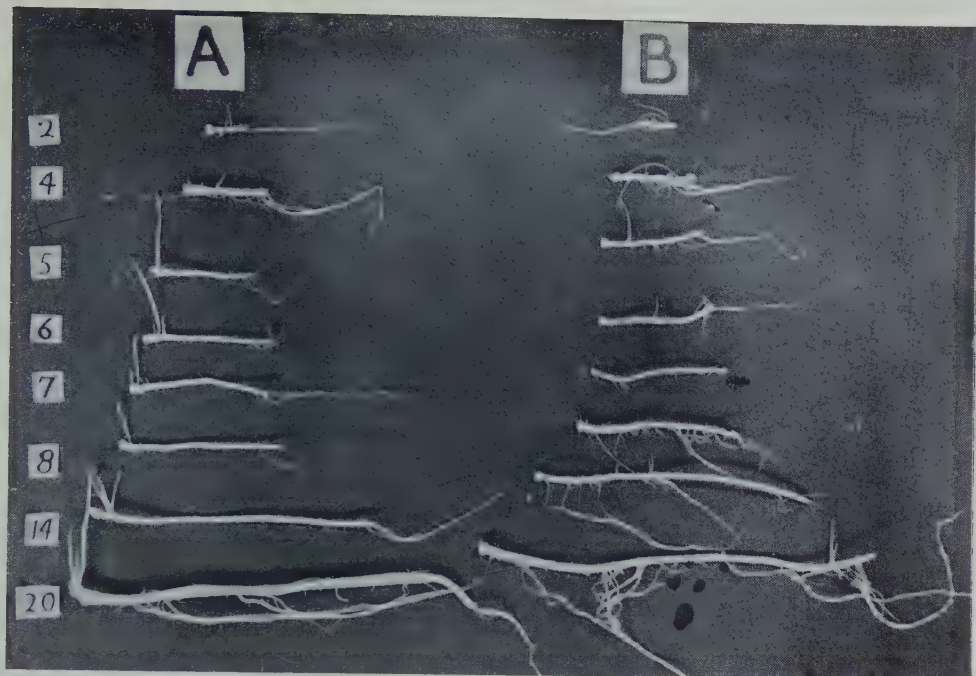
EXPLANATION OF PLATE VII

Illustrating the paper by J. Dore and W. T. Williams on Regeneration in Horseradish.

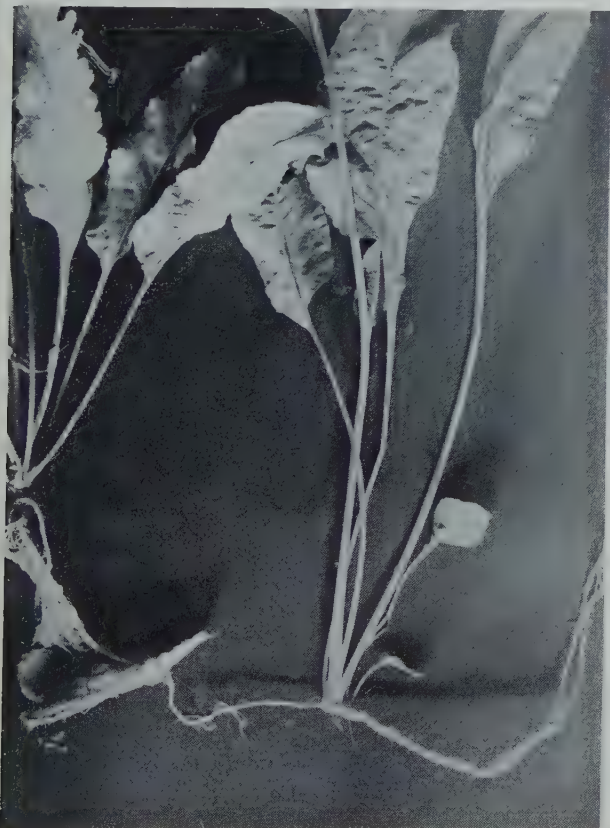
(i) Effect of proximally applied auxin on position of elongating bud. On left (A) control cuttings; on right (B) cuttings to which auxin paste has been applied at the proximal (left) end. Figures at left represent cutting lengths in cm.

(ii) Old crown (left) which has spontaneously produced a new crown at a distance of approximately 20 cm.

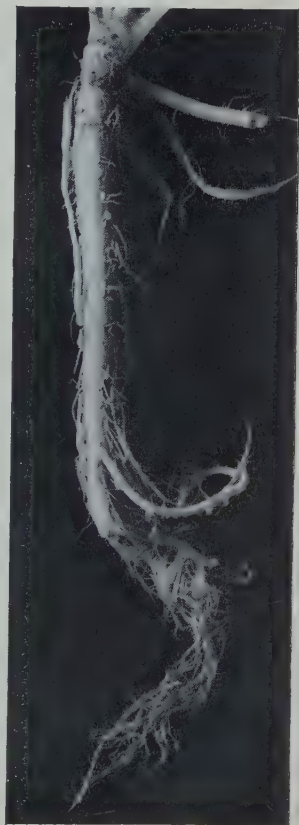
(iii) Tap root of potted plant which has regenerated spontaneously, apparently as a result of removal of lower part of root. Note that the new primordia are in vertical rows.



i



ii



iii

Distribution of Fungi in a Small Wood

BY

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ABSTRACT

Statistical analysis of the distribution of Basidiomycetes in a small wood (32 acres) shows significant features of non-uniform distribution. Some parts of this can be correlated with the age of the forest cover. A second noticeable feature of the non-uniform species is the apparent presence in all of aerial methods of spore dissemination and even of structures suggestive of adaptation to wind transport of the spores. It is nevertheless suggested that the facts imply slow dispersal in the non-uniform species and possibly a dependence on insects for dissemination.

INTRODUCTION

THE object of the work reported in this paper was to ascertain whether there is an appreciable number of species of the larger Basidiomycetes whose means of natural dispersal are such as to restrict them to as low a rate of spread as that enjoyed by many seed plants; and, if such species exist, to discern any taxonomic correlations which they may show. Although it is plausible that organisms with such small propagula as fungus spores should be rapidly dispersed by wind, the habit of many of them, especially resupinate species, does not seem adapted to this end. Moreover even among the agarics, which are definitely anemochoric, rapidity of dispersal is less in evidence than has often been believed.

To provide any convincing demonstration of the effect sought, it is necessary to find a sufficiently small area of woodland containing sufficient elements of ecological diversity to provide the initial foci for uneven distributions, and sufficiently young for them not to have been obliterated. Evidently an isolated uniform plantation is likely to acquire a statistically random distribution of colonizing fungi, as all the foci of infection will be relatively remote. But if, as in the case taken here, the plantation consists of stands of varying age, the older parts will provide a focus from which a given species can advance, slowly or rapidly, into the newer, and so could give rise, for some time at least, to statistically non-uniform distributions, which can be revealed in time by the collection of sufficient observations.

LOCALITY

The data presented here were collected in Madingley Wood, situated about 3 miles west of Cambridge. This is a plantation in which *Quercus*, *Fraxinus*, and *Ulmus* are dominant in different parts, growing on the chalky boulder-clay. The total area is 32 acres. The youngest parts of the wood consist

of self-sown *Fraxinus* about 50 years old, and the oldest parts contain mainly *Quercus*, and constitute the western half of the plantation.

MYCOLOGICAL METHODS

For the purpose of collecting the data, the wood has been divided into 26 arbitrary and unequal areas, delimited so as to allow for the readiest possible determination of the area in which each record falls without recourse to maps or measurements. Each area falls wholly within one or other of the age classes of the plantation. In making the observations it has been found extremely difficult to devise any satisfactory sampling technique which will give an unbiased estimate of the numbers of any species within any given area. The first difficulty is to find a way of comparing species of different habitat, when it is almost impossible to be sure that both are sampled with equal efficiency, or even that the relative efficiency of each can be estimated. For example, a resupinate and a dimidiolate species (for instance, *Tyromyces viridans* and *T. fragilis*) cannot be placed in the same class in regard to sampling efficiency, for it is inevitable that far more specimens of the former will escape the eye than of the latter, whose colour and posture make it relatively conspicuous. And one does not know, short of a complete quadrat count, the efficiency of sampling either of them. Quadrat counts moreover are scarcely practicable in the case of species, such as *T. viridans*, of which (at a guess) there may be only a dozen or so individuals in the whole plantation.

For want of a better method, therefore, the aim has been to cover every one of the 26 areas at regular intervals, and to estimate from the records the relative intensity of sampling in each. Since they are in any case unequal in area, the relative frequency of records from them will not be equal. But their relative contributions to the total can be estimated. Particular care was exercised to cover every type of habitat open to the fungi. All specimens were examined and identified, if not in the field in the laboratory, within 48 hours of gathering them. In the field notes, all records of each species which could reasonably be attributed to separate mycelia were severally noted, so that in principle one could tabulate the exact numbers of each species observed on each visit to each area. Actually, however, these figures are not very reliable, partly because of the difficulty of determining the limits of separate mycelia, and also because of the doubt existing, for the purpose of the present investigation, of the significance of a large number of records within a small area on the same day. This might bear witness to vegetative spread, or to sudden local dissemination in favourable weather by spores, or be an example of the species' normal mode of dispersal. For this reason, such local concentrations have been regarded as single records only. Conversely, two records at different times from the same area may in some cases have been due to a single mycelium; some cases of this can be confirmed, and these are of course noted, but there must be many cases which have escaped detection.

The period covered by the records employed here was from June 1952 to December 1954. From May 1953 every month is represented at least once. Observations have not been made in times of frost; otherwise weather conditions have not been allowed to affect visits to the wood, which are normally made once a week except in the coldest period of the year and in September.

RELATIVE SIZE OF POPULATIONS

A preliminary question, which must be disposed of before we can investigate the significance of any apparently non-uniform distribution, is to determine whether there exist significant differences between the species of different groups in the mean populations attained. Because, obviously, the larger the number of records of a species, the more likely is any apparent departure from a uniform distribution among them to be judged significant; and if any taxon is especially rich in abundant or in sparse species, that taxon is likely to be over- or under-represented among the species with non-uniform distributions.

Presumably, among the various species recorded, there will be a wide range of total populations. It is normally found that such populations are distributed according to the logarithmic series; that is to say, there exists a parameter x such that the number of species having a population of i individuals is proportional to x^i/i . This is in fact so generally observed as to be acceptable as an empirical law, the more reliable in that it can be related to theoretical expectations. We shall accordingly assume that it holds for all taxa of the Basidiomycetes considered in the present work. The logarithmic series can then be used to estimate the average population per species in different taxa, since this may be taken to depend on the mean of the distribution (that is, the mean number of records per species).

As already mentioned, however, the recorded numbers of observations of the various species are not a reliable guide to the number of individuals seen. On the other hand, we can reasonably rely on the distinction between those species which have been recorded once only, and those found more than once. The only source of error in this classification is the possibility that two records from the same area at different visits may refer to identical mycelia and so be equivalent to a single record for population purposes; the number of cases of this description is not large, and in some of them (where the fungus is growing on a particular log or tree stump) the case is proven. No serious error will result from making the same assumption in those cases where definite evidence is wanting.

In order to estimate the mean of a logarithmic series from the proportion y of the whole series contained in its first term, the following formulae are required:

$$y = -x/\log(1-x) \quad (1 a)$$

$$m = -x/(1-x)\log(1-x) \quad (1 b)$$

where m is the required mean. In order to estimate approximately the significance of these estimates, we can use the standard deviation of the estimate m obtained from the above formula by the method of maximum likelihood. Although the distribution is rather skew, the significances obtained thus will be sufficiently accurate to assess the results. The required formula is:

$$\sigma_m \sqrt{N} = \frac{\log(1-x) + x}{(\log(1-x) + x/1-x)(1-x)^2} \sqrt{\left(-\frac{x}{\log 1-x} - \frac{x^2}{(\log(1-x))^2} \right)} \quad (2)$$

where N is the total number of species in the series. In practice, the best way to use this formula is to plot the standard deviation as a function of m ,

TABLE I

Order or family	Number of species						Proportion with 1 record	
	With 1 record		2 or more		Total		with 1 record	
	<i>B</i>	<i>N</i>	<i>B</i>	<i>N</i>	<i>B</i>	<i>N</i>	<i>B</i>	<i>N</i>
Tremellales	12	13	14	16	26	29	0.461	0.448
Exobasidiales	2	2	2	2	4	4	0.500	0.500
Thelephorales	23	30	30	32	53	62	0.434	0.484
Polyporales	40	54	85	97	125	151	0.320	0.358
Meruliacei	3	4	6	9	9	13	—	—
Corticacei	14	16	41	45	55	61	0.254	0.262
Odontiacei	4	9	7	9	11	18	—	—
Polyporacei	19	25	28	31	47	56	0.404	0.446
Fistulinacei	0	0	3	3	3	3	—	—
Hymenochaetales	8	8	8	8	16	16	0.500	0.500
Agaricales	77	94	106	120	183	214	0.420	0.430
Clavariacei	4	4	8	8	12	12	—	—
Paxillacei	1	1	0	0	1	1	—	—
Hygrophoracei . . .	3	3	3	3	6	6	—	—
Tricholomatacei . .	32	40	42	52	74	92	0.432	0.435
Rhodophyllacei . .	6	6	2	2	8	8	—	—
Cortinariacei . . .	9	14	28	29	37	43	0.243	0.326
Coprinacei	11	15	20	22	31	37	0.355	0.405
Agaricacei	11	11	3	4	14	15	0.786	0.734
Russulales	11	11	2	2	13	13	0.846	0.846
Lycoperdales	0	1	1	1	1	2	—	—
Total of all orders . .	173	213	248	278	421	491	0.411	0.434

B = broad, *N* = narrow classification: see text.

using the formulae (1), for the case $N = 1$, from which the required values can easily be found.

The data, extracted from the field notes and tabulated for the purpose of this calculation, are given in Table I. In order to determine whether the results are in any way affected by the system of finer classification employed, the numbers of species have been entered twice, in one case using the broadest possible concept of specific circumscription (= broad = *B* in the table), and in the other using every named subdivision of these broad species as a separate entity—a narrow classification (*N* in the table). Two remarks on the higher classification are called for. First, the Tremellales are taken in the broad sense, including the Auriculariales and Dacrymycetales of some authors; second, the Thelephorales comprise mainly species of the family

Hypochnacei, which is here defined on the basis of hymenial structure to include species with clustered basidia and no subhymenium, a delimitation which carries into this family many species commonly included in *Corticium* and allied genera.

When the formulae given above are applied to the data in this table the results shown in Table II are obtained. Here again the figures refer to both narrow and broad delimitation of species. It is found that in no case does the use of the narrower delimitation make any significant difference, nor alter appreciably the significances of those in the table. This being so the broader delimitation is preferred, since its use makes the numbers of records per species greater and therefore more likely to attain significance in the distribution studies.

TABLE II

Taxon	Number of <i>B</i> species	Mean number of records per sp.			
		<i>B</i> species		<i>N</i> species	
		Mean	st. dev. of mean	Mean	st. dev. of mean
Tremellales	26	2.9	1.20	3.05	—
Exobasidiales	4	—	—	—	—
Thelephorales	53	3.25	—	2.70	—
Polyporales (all)	125	6.2	2.06	4.8	—
Corticacei	55	12.2	7.93	11.2	6.54
Polyporacei	47	3.7	—	3.1	0.96
Hymenochaetales	16	2.45	1.00	2.45	1.00
Agaricales (all)	183	3.45	—	3.17	—
Tricholomatacei	74	3.25	—	3.15	—
Cortinariacei	37	14.0	10.69	5.9	3.24
Coprinacei	31	4.85	—	3.75	—
Agaricacei	14	1.30	0.20	1.40	0.28
Russulales	13	1.20	0.11	1.20	0.11
Lycoperdales	1	—	—	—	—
Total of all orders	421	3.60	0.50	3.25	0.365

From inspection of these results it appears that the mean number of records per species in the Agaricacei and the Russulales is significantly less than the general mean. No other single taxon differs significantly from the general mean, but the Polyporales are more often recorded than either of the above-mentioned groups, and less clearly so ($P = 0.12$) than the Hymenochaetales. The latter difference is mainly due to the Corticiacei, but the figures for this family are not separately significant. The implication is that differences in numbers of records are in some cases correlated with taxonomic position.

This conclusion cannot be at once extended to actual populations, since different taxa differ in their appearance and habits, and therefore in the efficiency with which they are sampled. The larger humicolous species are in particular likely to be more efficiently sampled than the smaller and lignicolous ones. Since the two groups distinguished by small populations are both on this basis likely to be over-represented in the records, the reality

of their difference is confirmed. Conversely, the relatively populous Corticiacei are likely to have the same sampling efficiency as other resupinates, and a lower one than the Hymenochaetales, most of which are large bracket polypores easy to observe. It is therefore safe to conclude that real differences in the relative populations of species exist between orders and families.

DISTRIBUTION OF PARTICULAR SPECIES

As previously mentioned, the whole plantation has been divided for purposes of the field records into 26 unequal areas. Not only are these unequal in area, but also in accessibility. It follows that they have to be given unequal weights in assessing the frequency of records in each. To each area was therefore assigned a weight from 1 to 10, determined by the relative frequency of total records; the sum of all the weights was 144.

If now a given species has been recorded only from certain areas, which together constitute a compact group, we can compute the probability that such a concentration would arise by chance if the same number of records were scattered at random throughout the wood. If the sum of the weights of the included areas be a , and the number of records of the species be n , then the required probability is

$$P(n, a) = \left(\frac{a}{144} \right)^{n-1} \quad (3)$$

If the records are not distributed in contiguous areas, they may still of course be attributed to a contiguous group by admitting intervening areas which have no records. It is also possible that a species might show a significant concentration of records in two or more separate regions; but in fact, owing to the limited resolving power of the observations, no case of this reaches significance.

The whole species list was gone through, and all those species for which P calculated as above was not greater than 0.05 were listed. There were 45 such species. Now one would expect that among a total of 421 species, about 21 would by chance distribution exhibit a value of P as small as this. We may therefore conclude that there are about 24 species which exhibit a significantly non-uniform distribution by the criteria employed. This is, of course, a lower limit to the number of species which have in reality such a non-uniform distribution, since there are many ways in which such a species might escape our list (no species can enter with less than two records, for example), whereas, even if there were no such omissions, the lower fiducial limit (at 5 per cent.) of the number of non-uniformly distributed species is 15.

It should be mentioned that, in order that the uncertainty referred to in the exact numbers of records should not bias the results in the wrong way, the number used for n in these calculations was in each case the lowest number consistent with the field records.

In order further to assess the significance of the list, the species were

classified in four groups, according to the probability of their genuineness. In Group I were placed those species which are independently significantly non-uniform in distribution; the criterion for this being that $421 \cdot P$ is not greater than 0.05. In Group II come those which are more likely than not to be genuine members of the list, for which $421 \cdot P$ is not more than 0.5. In Group III are placed the remaining species, in order of increasing P , required to make up the estimated number of 24. Further consideration shows that these three groups do not exhaust the species which require to be considered. For if we assign a weight to each, proportional to the probability that it is not an ordinary species, the weights will be equal to $\exp(-421 \cdot P)$; and there are a few species left whose weights collectively are not negligible. We therefore include in Group IV as many further species as are needed to bring the aggregate weight of the remainder below 0.0005 (i.e. negligible to three places of decimals). It turns out that five additional species fall into this group. Although we now have a list of 29 species, we still expect that the most likely number of genuinely non-uniform species is 24; we therefore apply a scale factor to the weights so that their sum for all 29 species comes to 24, and in subsequent calculations use sums of these weights in place of actual numbers of species. In this way we have allowed for the inevitable uncertainty attached to drawing up such a list. The list finally arrived at is given in Table III.

CORRELATION WITH TAXONOMY

In order to discover whether there is any significant tendency for some taxa to be better represented than others among the species with non-uniform distribution, it is not sufficient to compare their numbers in the above grouping with their representation in the whole species list. It is necessary to make allowance for the fact that a species of small population is for that reason less likely to provide enough records to qualify for inclusion in the above groups, even if it is in reality of non-uniform distribution. And since we have already shown that the species of some taxa possess larger populations in the plantation studied than others, this factor will produce a bias as between taxa.

To correct for this, we need to know how the chances of inclusion in the list of non-uniform species depends on the number of records. It will be assumed that this follows the equation:

$$p(n) = 1 - \exp(-b(n-1))$$

where n is the number of records, and b is a parameter, which can be estimated from the fact that the mean value of p for all n is known to be $24/421$. But also

$$\bar{p} = 1 - e^{-b} \cdot \frac{\log(1 - xe^{-b})}{\log(1 - x)} \quad (4)$$

TABLE III

Name of Species	Order or family	Region of occurrence	Habitat	1/P	Weight
Group I					
<i>Hymenochaete corrugata</i> (Fr.) Lév.	Hym.	West Cen.	Lign.	1×10^{10}	1.79
<i>Peniophorella pubera</i> (Fr.) Karst.	Cortic.	Western	Lign.	4×10^6	1.79
<i>Marasmius ramealis</i> (Bull. ex Fr.) Fr.	Trich.	Western	Quisq.	2×10^5	1.79
<i>Daedalea confragosa</i> (Bolt.) Fr.	Polyp.	Central	Lign.	9×10^4	1.77
<i>Corticium tuberculatum</i> Karst.	Cortic.	Western	Lign.	1×10^4	1.72
<i>Daedalea quercina</i> (L.) Fr. . .	Polyp.	Nth. West.	Lign.	9×10^3	1.70
Group II					
<i>Entoloma lividum</i> (Bull. ex Fr.) Kumm.	Rhod.	West Cen.	Humus	7,776	1.70
<i>Hymenochaete rubiginosa</i> (D. ex Fr.) Lév.	Hym.	Sth. Cen.	Lign.	3,300	1.58
<i>Botryobasidium pruina- tum</i> (Bres.) Donk	Thel.	Nth. West.	Fungi	3,014	1.56
<i>Dacrymyces deliquescens</i> (Bul.) Duby	Trem.	Western	Lign.	2,510	1.52
Group III					
<i>Exidia thuretiana</i> (Lév.) Fr. .	Trem.	Nth. West.	Lign.	608	0.91
<i>Prillieuxia umbrina</i> (Fr.) c.n.	Thel.	Sth. Cen.	Lign.	602	0.90
<i>Mycena ammoniaca</i> (Fr.) Quél.	Trich.	Eastern	Humus	526	0.82
<i>Fomitopsis pinicola</i> (Sow. ex Fr.) Karst.	Polyp.	NW. Cen.	Lign.	424	0.66
<i>Pleurotus cornucopiae</i> (Paul ex Fr.) P. & D.	Polyp.	Nth. East.	Lign.	372	0.57
<i>Hypoloma fasciculare</i> (Huds. ex Fr.) Kumm.	Cortin.	Sth. West.	Lign.	361	0.55
<i>Protodontia livida</i> (Bres.) c.n.?	Trem.	Sth. East.	Lign.	282	0.39
<i>Athelia byssinella</i> (Bourd.) c.n.	Thel.	Sth. East.	Lign.	282	0.39
<i>A. mutabilis</i> (Pers. ex. Fr.) c.n.	Thel.	Sth. East.	Lign.	282	0.39
<i>Pluteus cervinus</i> (Schaeff. ex Fr.) Fr.	Agar.	Sth. West.	Lign.	282	0.39
<i>Inocybe geophylla lilacina</i> Fr.	Cortin.	West Cen.	Humus	268	0.37
<i>Cortinarius decipiens</i> Fr., ss. K. & R.	Cortin.	West Cen.	Humus	208	0.23
<i>Terana laevis</i> (Pers. ex Fr.) c.n.	Cortic.	Nth. West.	Lign.	$191\frac{1}{2}$	0.20
<i>Mycena vitilis</i> (Fr.) Quél., nec Kühn.	Trich.	West Cen.	Humus	$191\frac{1}{2}$	0.20
Group IV					
<i>Mycoacia uda</i> (Pers. ex Fr.) Donk	Odont.	Eastern	Lign.	133	0.075
<i>Cortinarius uraceus</i> Fr. . . .	Cortin.	SW. Cen.	Humus	122	0.057
<i>Phanerochaete chordalis</i> (Höhn & Litsch.) c.n.	Cortic.	Sth. West.	Lign.	83	0.011
<i>Clavaria acuta</i> Sow. ex Fr. .	Clav.	Eastern	Humus	$76\frac{1}{2}$	0.007
<i>Merulius lividus</i> (Pers. ex Fr.) c.n.	Merul.	Western	Lign.	$67\frac{1}{2}$	0.004

and as we know the values of $\bar{p}$ and x for the whole series, we can find b . It turns out that $b = 1/41.2$; it follows that

$$p(n) = 1 - \exp((1-n)/41.2) \quad (5)$$

If now in a given taxon the numbers of records per species are distributed in the logarithmic series with a parameter x , the expected representation of that taxon in the list of non-uniform species will be

$$S' = -S \cdot \sum_{n=1}^{\infty} p(n) \cdot x^n / n \log(1-x)$$

which, on substituting from (5) gives us

$$S' = S \cdot \left(1 - \frac{1.0254 \log(1-0.9756 \cdot x)}{\log(1-x)} \right)$$

where S is the total number of species in the taxon recorded from the wood examined, and S' is the number of these species which, if there were no bias in this respect between different taxa, would be expected among the

TABLE IV

Order or family	Numbers of non-uniform species		
	Expected	Observed (sum of weights)	P
Tremellales	1.22	1.58	—
Thelephorales	2.62	1.82	—
Polyporales (all families)	9.02	4.78	0.04
Corticiacei	4.74	2.09	0.07
Polyporacei	2.78	2.65	—
Hymenochaetales	0.53	1.89	c. 0.35
Agaricales (all families)	10.19	3.43	0.003
Tricholomatacei	3.66	1.57	0.1
Cortinariacei	4.05	0.68	0.05
Coprinacei	1.19	0	(0.3)
Agaricacei	0.29	0.22	—
Russulales	0.07	0	(0.45)
Taxa with large populations	9.26	4.96	0.09
Taxa with medium populations	13.60	13.71	0.5
Taxa with small populations	1.14	5.49	0.028

(In the second part of the table the taxa (including those not referred to in the first part) are classified in a manner described in the next section.)

non-uniformly distributed set of species. It is evident that the data of Table II enable us to calculate S' from this formula for each of the taxa there considered. The results of this calculation are given in Table IV; beside the expected numbers of species out of each taxon in the non-uniform list are entered the sums of the weights of the species actually present, from Table III, which, as we have seen, is the proper figure to compare the expectations with. In the third column are entered the probabilities that so great a difference of the observed sign will be produced by chance, wherever the significance of the difference is in question.

The χ^2 -value calculated from the first part of Table IV is definitely significant, implying that there is indeed a difference between the taxa in regard to their representation among the non-uniform species. In particular it appears that the Polyporales (especially the Corticiacei) and the Agaricales (especially the Cortinariacei) are under-represented, indicating that the members of these groups are on the whole adapted for wide dispersal. None of the taxa mentioned is significantly over-represented in the non-uniform list.

CORRELATION WITH SPECIES POPULATION

In order to discover what correlation there might be with the varying species population numbers, which were found above to differ as between taxa, the taxa enumerated in Table I were divided into three classes. Those for which the average records per species were greater than the general mean at a significance level of 50 per cent. (for which the difference is more likely than not to be real) formed the first, those with fewer records per species than the general mean, at the same significance level, formed the third, and the remainder were placed in the second class. All taxa were included in this enumeration; it may be rather arbitrary to say that the Lycoperdales in Madingley Wood have large populations, on the grounds that the single species has been recorded more than once: but then a single species will not greatly disturb the results either way. It is less justifiable to drop certain species from the classification merely because they keep statistically bad company.

The expectations were obtained by adding those for the included taxa within each of the three classes. The results are given in the second part of Table IV. It will be seen that there is a marked correlation. The taxa with small populations are markedly over-represented among the non-uniform species, and those with large populations appreciably under-represented. This is of course as would be expected; whatever factors are responsible for the retardation of dissemination in those species which remain confined to certain parts of the wood are likely to prevent the establishment of large populations. Particular interest therefore attaches to those species which remain in a confined area in spite of a large population: this applies to all the species in Group I of Table III, except the two *Daedaleae*.

CORRELATION WITH HABIT AND HABITAT

The species can also be classified, without reference to taxonomy, according to their habitat and habit of growth. One might expect to find a strong correlation between these characters and speed of dissemination; those species which habitually grow in confined spaces under rotting logs, for example, would seem to have less opportunity for the more rapid modes of dispersal than those producing pileate fructifications in open places. Six types were recognized under this head. These are (i) collomycetes, i.e. species with hymenial gel and hydrophobic spores and no geotropic orientation;

(ii) resupinates; (iii) dimidiates; (iv) anomalous species with no hymenial gel but with hymenium often facing upwards or precariously vertical, often of special habitats; (v) lignicolous-pileate species, or dimidiates growing in high places; (vi) humicolous species with well-exposed hymenium.

The statistical data concerning these six habit-classes are presented in Table V.

TABLE V

Habit	Number of species			proportion 1 record	mean records per species	non-uniform species		P
	with 1 record	2 &c. records	Total			expected	observed	
Collomycetes	3	7	10	0.300	7.16	1.23	2.43	0.3
Resupinates	64	104	166	0.374	4.16	9.69	9.18	0.5
Dimidiates	14	24	38	0.368	4.26	2.51	5.73	0.15
Anomalous	6	5	11	0.545	2.06	0.26	0	0.45
Lignicolous pileate	16	40	56	0.286	8.23	5.47	3.82	0.25
Humicolous pileate	68	72	140	0.486	2.61	4.84	2.86	0.3

Although the χ^2 -value for the whole table is just significant, there is no single habit-class for which there is a separately significant discrepancy between the expected and observed numbers of non-uniform species. The most suggestive case is that of the dimidiates, which may be over-represented among those with restricted distributions. Certainly there is no confirmation of the suggestion that resupinates might be over-represented. There is no clear indication that habit, as here defined, is a factor determining these non-uniform distributions.

CORRELATION WITH AGE OF WOODLAND

As already mentioned, the oldest part of the plantation is that in the west. There is a second group of older areas in the eastern half of the wood, originally planted as windbreaks, which form a cross-shaped region dividing the eastern half into four parts. On account of the high ratio of perimeter to area for this region, it is hardly to be expected that even the most slowly dispersed species will have remained within it for upwards of 30 years. The only opportunity for an effective comparison between older and younger areas is therefore to separate the western from the eastern parts. Since a number of species in fact occupy regions covering both sides of the boundary but not extending to the extreme east or west, it is necessary to recognize a third, intermediate, region, containing all the areas which abut on the east-west boundary.

In view of the way in which the areas have been weighted, we ought to find that among the species credited with non-uniform distribution, the numbers belonging to each of these three regions should be proportional to the sums of the weights of the areas composing them; provided that all regions are equally likely to possess 'endemics'. In fact, the results are as presented in Table VI.

It will be observed that there is a significant excess of western species, and a significant defect of eastern ones, in the non-uniform list. This is in

fact the most significant correlation observed. We must therefore conclude that most of the weight of the non-uniform list consists in species found in the older, western, part of the plantation. This is, of course, in accordance with common-sense expectation; it is therefore worth remarking that it is not the only possible explanation of the effect. If, for example, the fungus population of the wood were derived in the main from remote sources, the prevalence of westerly winds might produce an excess of recent introductions on the western side of the wood, even if it had been all of one age.

TABLE VI

Region	Sum of weights of included areas	Number of non-uniform species		P
		expected	observed	
Western	62	7.78	15.19	0.02
Central	40	5.04	4.18	—
Eastern	89	11.18	4.63	0.03

GENERAL DISCUSSION

One fact clearly emerges from the foregoing analysis; that there are in Madingley Wood, and therefore presumably elsewhere in similar situations, a number of species of basidiomycetes whose methods of dispersal in nature, whatever they may be, are such as to impose on them a quite slow rate of dissemination over small areas. There are species there which have presumably been present in the western half of the wood ever since the eastern part was allowed to revert to woodland about 50 years ago, and which have nevertheless not advanced as much as a furlong from their original home. The evidence does not allow us to conclude that there are any species which possess a more rapid rate of spread, since all those species which are in fact distributed throughout the present area of the plantation may have come to be so through repeated remote infection by wind-borne spores, and not spread within the wood at all. In view of the ecological history of the wood, let alone general probability, this is, however, very unlikely. That the number of slowly dispersed species is in fact much greater than the 24 which we have estimated as being demonstrably non-uniform is very probable; but it is unlikely that they form a majority of the 421 species in the full list.

There are, however, also some species which are probably if not undoubtedly of restricted distribution, but which are not found in the older parts. The restricted distribution of these may perhaps be determined ecologically; that is, their conditions of growth may not be realized in the more mature forest conditions of the western half of the plantation. It is very improbable that this purely ecological interpretation could be advanced for the confinement of certain species to the western half, because of all these only one, *Inocybe geophylla*, is not a lignicolous species, and the availability of suitable lignicolous habitats in the eastern half is at present no less than in the west. But the possibility does exist for humicolous species. Great changes certainly occur in the structure and composition of humus during the maturation of

forest communities, and these proceed quite slowly. It would therefore appear worth while to consider the effect of abstracting from the species considered in Table V all those having an eastern distribution. According to this hypothesis, this procedure ought to enhance the general significance of the habit correlations, and we should expect that in particular the observed defect of humicolous species should be intensified. The amended table appears as follows, and should be read in conjunction with Table V:

TABLE VII

Habit		Total weight of species eliminated	Emended numbers of non-uniform species	
			expected	observed
Collomycetes	. . .	0	0.84	2.43
Resupinates	. . .	2.06	6.64	7.11
Dimidiates	. . .	2.33	1.72	3.37
Anomalous	. . .	0	0.18	0
Lignicolous	. . .	0.97	3.75	2.84
Humicolous	. . .	2.15	3.31	0.69

From this it may be inferred that, of those species whose distribution is limited to the younger parts of the wood, the majority are of humicolous habit. This accords also with the observation that the Agaricales, to which order the greater part of the humicolous species belong, are strongly deficient among the observed non-uniform species. On the other hand, the Polyporales, mostly lignicolous, are almost equally deficient. It is perhaps significant here that these two are the largest and most successful orders of Eubasidiomycetes not only in this locality but in the world. We have also found that the species with non-uniform distribution tend to have smaller populations than those more evenly spread, or, more exactly, to belong to taxa characterized by smaller populations. If we can in general equate the non-uniform species with those which have relatively slow methods of natural dissemination, these facts together suggest that tardy distribution links up not only to limited spread of the species, but to a more limited radiation in the evolution of larger taxa.

The question must finally be considered, what is the means of natural spread which, notwithstanding the apparent capacity of many of the species concerned for anemochoric dissemination, results in so slow a progress? With many lignicolous fungi, insects certainly play an important part in their general ecology, and may in some manner serve as vectors for dissemination also. As against this, it must be observed that none of these species has any obvious adaptation for such a mode of life; and that zoochory is in plants generally associated with rapid evolution and taxonomic innovations, whereas in the present case the affected species tend to belong to supposedly 'primitive' groups. That some of the humicolous species owe their restricted distribution to strictly ecological factors has already been suggested, and it is possible that microclimatic or other differences may be sufficient to account for the restriction of other species in the same way; but, on the

basis of such observations as have been made (from which meteorological measurements have been omitted), this seems to be far-fetched. No doubt different factors operate in different cases. Entomochory remains in my view the most probable hypothesis (though it is unlikely to be a necessary factor in the actual translocation of basidiospores, except perhaps in resupinates; possibly these spores are unable to establish mycelia of themselves in nature, and serve only as organs of comportation between entomochorically established mycelia?). But obviously there is a large problem here which, since it concerns apparently many of the sort of fungi which are serious pests of forestry, is likely to be of considerable importance. How, in nature, do wood-destroying basidiomycetes get about? Laboratory experiments cannot of themselves give the answer to this question.

SUMMARY

Observations are recorded on the distribution of species of Basidiomycetes within Madingley Wood, near Cambridge. Statistical methods applied to the data indicate that out of a total of over 400 species recorded up to the end of 1954 some 24 are likely to be significantly non-uniform in their distribution, that is to say, confined within the wood to certain parts of it.

All these species are provided with means of aerial dissemination in the form of basidiospores; in many of them the hymenophore is exposed in such a way as to suggest specific adaptation to anemochoric transport. Such a mode of dissemination should rapidly bring about the uniform spread of such a species over the whole of so small an area (about 32 acres). That this has not apparently occurred, in so many cases, poses a problem.

Further statistical calculations have shown that this non-uniform distribution is a habit correlated with various other factors. In taxonomy, certain orders are significantly under-represented: these are the two largest, the Polyporales and the Agaricales. Taxa whose species have small populations within the wood, compared to the general mean, are over-represented, and those with populous species are under-represented. As to age of forest cover, which varies from place to place in the wood, most of the non-uniform species favour the oldest parts, and the smallest contribution to the list comes from the youngest stands.

All these facts tend to show that the non-uniform species are in fact subject to slow rates of dispersal, and for this reason have not spread over the whole area; though there are a few cases where the restriction may be attributed to ecological factors only. It is suggested that insects may play an essential part in the dissemination of some species, but the apparently anemophilous adaptations of many of them are puzzling. The possibility that basidiospores may sometimes be mainly agents of comportation is worth consideration.

Photosynthesis and Formation of Fats in a Diatom

BY

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ABSTRACT

A study has been made, using C^{14} as a tracer, of the variations which occur according to the physiological condition of the cells in the distribution of carbon fixed, both in the light and in the dark, by the diatom *Navicula pelliculosa* (Bréb.) Hilse during periods of 30 to 300 seconds. Fixation into the following cell fractions was determined: (A) material soluble in 80 per cent. ethanol but insoluble in benzene, (B) material soluble both in 80 per cent. ethanol and in benzene, and (C) material insoluble in 80 per cent. ethanol. Carbon fixed in photosynthesis was incorporated, rapidly and in amounts representing up to 70 per cent. of the total fixation, into fractions B and C, as well as into fraction A. Considerable variation was found in the proportions of carbon entering the three fractions in the light; in actively growing cells the proportion entering fraction C preponderated over that in B, corresponding to the synthesis of protein, whereas in nitrogen-deficient cells fixation in B was the greater, corresponding to the synthesis of fat. These patterns changed only slowly, over periods of days, following the transfer of cells to altered conditions of nitrate supply. However, when ammonium nitrogen was supplied to nitrogen-deficient cells a marked change in distribution of carbon fixed occurred within 5 minutes, fixation in fraction B falling to a low value and that in A rising correspondingly. In cells subjected to prolonged nitrogen-deficiency, fixation in fraction B fell to a relatively low value but the proportion which this fraction formed of the total dry matter in the cells rose as a result of an increased rate of loss from the cells of constituents other than lipides. The distribution of carbon fixed was also dependent on light intensity. Fixation in fractions B and C rose relatively to that in A as light intensity was increased up to 100 foot-candles but fell again at the highest intensity used, 2,000 foot-candles. These results are discussed with particular reference to the relationship between fat accumulation and photosynthesis in algae.

INTRODUCTION

FOLLOWING the work of Sachs (1862) it has generally been held that the product of photosynthesis is carbohydrate, in the sense that the carbon assimilated is converted to this form before being elaborated, by processes independent of those involved in photosynthesis, to other types of compound. The evidence for this belief, which is still implicit in much thinking about photosynthesis, has been reviewed by Rabinowitch (1945). It lies chiefly in the constancy with which measurements of the photosynthetic

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quotient have yielded a value of approximately unity and, to a lesser extent, in the equivalence which is found between carbon dioxide assimilated and carbohydrate appearing in photosynthesizing organs. However, such determinations have almost invariably been made with systems in which carbohydrate synthesis might be expected to predominate over other processes, i.e. with algae held at high light intensities in media unsuitable for growth or with mature leaves.

Beijerinck (1904) interpreted the accumulation of fat in diatoms as the result of photosynthesis continuing in cells unable to divide, so that photosynthetic intermediates, which would otherwise be used for protoplasmic synthesis, become diverted to the formation of reserve materials. This idea was further developed by Myers (1949) who found evidence that in *Chlorella* the accumulating products of photosynthetic metabolism may vary greatly with the previous history of the cells and who postulated that photosynthesis and nitrogen metabolism share common intermediates and intermesh at some stage prior to the formation of carbohydrate. This hypothesis has been confirmed by the discovery that the carbon dioxide fixed in photosynthesis appears first in phosphoglyceric acid (Benson, Bassham, Calvin, Goodale, Haas, and Stepka, 1950; Fager and Rosenberg, 1952), for this intermediate is known to be readily transformable into substances implicated in a variety of metabolic sequences and there seems to be no good reason to suppose that in photosynthesis it must be converted exclusively into carbohydrate. Although it seems probable that the two-carbon fragment from which phosphoglyceric acid is formed by carboxylation is itself formed from phosphoglyceric acid by a cycle in which the intermediates are carbohydrate derivatives, these substances need not be involved in more than catalytic amounts. That carbon fixed in photosynthesis is, in fact, rapidly incorporated into fractions of *Scenedesmus* other than carbohydrate has been demonstrated by Brown, Fager, and Gaffron (1948) and Clendenning (1950).

There are thus good grounds for expecting that the products in which the carbon fixed in photosynthesis accumulates may be different according to circumstances, since the relative rates of the different synthetic processes competing for the primary product will vary with the previous history of the organism and the conditions to which it is exposed. Ouellet and Benson (1952) have shown that the hydrogen-ion concentration of the medium has considerable effects on the distribution of tracer carbon in photosynthesizing *Chlorella*, not only between total 80 per cent. alcohol-soluble and -insoluble fractions but also among various 80 per cent. alcohol-soluble substances. Variation with light intensity of the distribution of photosynthetically assimilated carbon among a variety of water-soluble substances has also been reported (Calvin, Bassham, Benson, Lynch, Ouellet, Schou, Stepka, and Tolbert, 1951).

The object of the investigations described in this paper was to determine, in a more general way, the effects of nitrogen nutrition on the distribution of carbon fixed by photosynthesis in a diatom, *Navicula pelliculosa*. It has

been difficult to reconcile our meagre knowledge of the metabolism of these organisms with the hypothesis that a carbohydrate is the product of photosynthesis in the sense defined above, for, whereas accumulation of fat often occurs in diatoms, starch, for example, is always absent and other carbohydrates are not conspicuous. Barker (1935), however, found the photosynthetic quotient of two species of *Nitzschia* to approximate to unity at both high and low light intensities and deduced from this that fat metabolism is not on the main line of synthetic activity in diatoms, although he was unable to demonstrate in them the presence of more than traces of the sugars which he expected as the primary products of photosynthesis. Nevertheless, recent work (Stosch, 1951; Collyer and Fogg, 1955) suggests that diatoms may contain substantial proportions of polysaccharide and there appears to be no compelling reason for supposing that their photosynthetic mechanism differs in any fundamental way from that in other algae and higher plants. A diatom was not included in a recent comparative study of short-term photosynthesis in plants belonging to various different major groups (Norris, Norris, and Calvin, 1955) but the results obtained showed that there is remarkable qualitative uniformity in the early products of photosynthesis and it would be surprising were diatoms found to be different in this respect. However, apparently as a result of decreasing cell nitrogen content, diatoms accumulate high proportions of fat (Beijerinck, 1904; Collyer and Fogg, 1955) and, as will be shown in this paper, under these circumstances a major part of the carbon fixed in photosynthesis appears to be assimilated directly to fat.

MATERIAL AND METHODS

Culture of the diatom

A strain of *Navicula pelliculosa* (Bréb.) Hilse, isolated by Lewin (1953) and already used in studies of fat accumulation (Collyer and Fogg, 1955), was grown in bacteria-free culture in 500 ml. portions of medium, continuously shaken and aerated with 4 per cent. carbon dioxide in air. The light intensity was about 800 foot-candles and the temperature $21 \pm 1^\circ \text{C}$. The medium was No. 10 of Chu (1942) with twice the normal amount of calcium nitrate, iron supplied as ferric citrate (Rodhe, 1948), and 2 per cent. by volume of soil extract. From a small inoculum, growth reached its maximum in about one week. At this stage the packed-cell volume (determined by centrifuging at 1,200 g for 20 minutes, all values given being the mean of duplicate determinations) was of the order of 0.005 ml. per ml. of medium. The stage of growth at harvesting varied according to the needs of individual experiments. Cells were harvested by centrifuging at 1,500 g for 5 minutes and resuspended by means of a spinning nylon test-tube brush. The basic medium used for resuspending was Chu No. 10 without nitrate or soil extract (subsequently referred to as 'nitrogen-free medium'). This preparation of material was not carried out under strictly aseptic conditions but microscopical examination showed that contamination was negligible even when suspensions prepared

in this way were incubated under conditions suitable for growth of the diatom for as long as 7 days.

Conditions of tracer fixation

Each experiment was carried out at a constant temperature within the range 18 to 23° C. 5 ml. samples of diatom suspension were exposed in flattened tubes presenting a thickness of 0.5 cm. of suspension to the incident light, which was of a standard intensity of 2,000 foot-candles from a tungsten projection lamp. The tube containing the suspension was water-cooled and air containing 4 per cent. of carbon dioxide was passed through it at the rate of about 3 bubbles per second. A preliminary illumination period, without tracer, of 5 minutes was allowed for steady state conditions to be attained. Tracer was injected as 0.5 ml. of 0.03 M $\text{Na}_2\text{C}^{14}\text{O}_3$ (about 5×10^5 counts per minute when measured as CaCO_3 as described below). The C^{14} was obtained as barium carbonate from the Isotopes Division, U.S. Atomic Energy Commission. Bubbling of the carbon dioxide-enriched air was continued for 30 seconds after this addition, giving a final pH of about 8.7. At the end of the exposure period (120 seconds in most experiments) the suspension was poured into boiling ethanol sufficient to give a final concentration of 80 per cent. of ethanol by volume. Parallel experiments in which the diatom suspensions were kept in the dark throughout, but under otherwise similar conditions, were made in addition.

Analytical methods

Following boiling of the cells in 80 per cent. ethanol for 4 minutes, fractionation was carried out by a procedure similar to that of Brown, Fager, and Gaffron (1948). The suspension was cooled and filtered through a tared sintered glass filter covered with a thin layer of kieselguhr to facilitate complete recovery of insoluble algal material. The residue on the filter (fraction C) was washed with 20 ml. of 80 per cent. ethanol containing 0.28 ml. of concentrated hydrochloric acid, then dried at 105° C. to constant weight. Filtrate and washings were combined and evaporated to dryness at room temperature in a current of air, thus removing unfixed tracer. The solid residue was extracted successively at room temperature with small portions of benzene and the combined extracts made up to 10 ml. (fraction B). The residue from the benzene extraction was similarly extracted with 80 per cent. ethanol and the combined extracts made up to 10 ml. (fraction A). The three fractions obtained were thus:

A. Ethanol soluble—benzene insoluble material; pale yellow in colour and forming less than 17 per cent. of the total dry weight of the cell material.

B. Ethanol soluble—benzene soluble material; olive green in colour and forming 15 to 35 per cent. of the total dry weight; containing the pigments and fatty materials.

C. Ethanol insoluble material; a white powder forming 65 to 75 per cent. of the total dry weight; consisting chiefly of proteins, polysaccharides, and silica.

The radioactivity of these fractions was determined after wet combustion to give calcium carbonate according to the procedure followed by Fager (1954). Samples of 27 ± 1.4 mg., 1.0 cm.² in area, were counted in a gas-flow counter. All counts reported are calculated total values for each fraction for tracer of standard activity (150,000 counts per minute per ml. of final suspension) and corrected for background count. Geometry and self-absorption were constant throughout. The standard deviations given in the tables and used in assessing the statistical significance of the results are those for counting error only.

Total nitrogen contents were determined using a conventional micro-Kjeldahl technique with a selenate catalyst in the digestion.

RESULTS

The time-course of fixation in actively growing and in nitrogen-deficient cells

Actively growing cells were obtained by adding an equal volume of fresh medium to a 5-day-old culture and harvesting 2 days later. The cells were washed in nitrogen-free medium, resuspended in medium containing nitrate, and samples exposed to tracer for periods ranging from 30 to 300 seconds. The observed changes in the radioactivity of the three cell fractions with time are shown in Fig. 1.

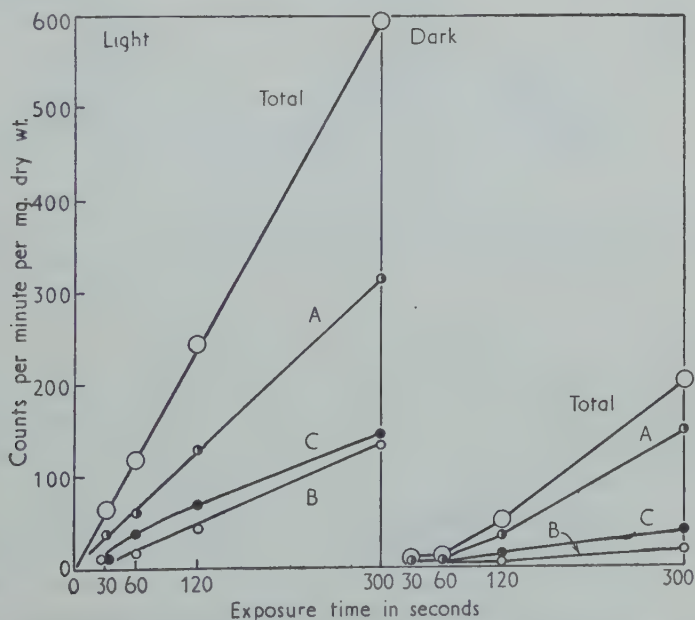


FIG. 1. Time course and distribution of fixation by actively growing cells of *Navicula pelliculosa* of C^{14} supplied as bicarbonate in the light and in the dark.

Suspension with 0.0022 ml. cells/ml. and 0.31 mg. dry wt./ml.; N content of cells 6.4% of dry wt.; N supplied as 0.25×10^{-3} M $Ca(NO_3)_2$. Radioactivity expressed as counts/minute/mg. dry wt. of cells. ○, total fixation; ●, fixation in fraction A (soluble in 80% ethanol, insoluble in benzene); ○, fixation in fraction B (soluble in 80% ethanol, soluble in benzene); ●, fixation in fraction C (insoluble in 80% ethanol).

In the light total fixation of tracer increased linearly with time (Fig. 1). In the individual fractions the increase was less exactly linear with time but the lines of best fit do not pass through the origin and indicate proportionately greater fixation in fraction *A* and less in fractions *B* and *C* in the first 30 seconds than was found with longer exposure periods. This is more evident when the activity of the fractions is plotted as a percentage of the

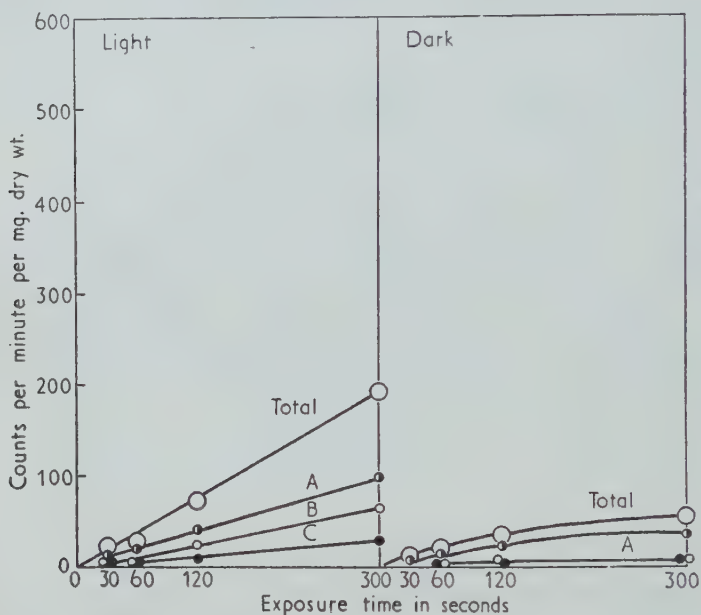


FIG. 2. Time course and distribution of fixation by nitrogen-deficient cells of *Navicula pelliculosa* of C^{14} supplied as bicarbonate in the light and in the dark.

Suspension with 0.021 ml. cells/ml. and 1.8 mg. dry wt./ml. Medium 'nitrogen-free'. Radioactivity expressed as counts/minute/mg. dry wt. of cells. Symbols as in Fig. 1.

total for the algal material (Fig. 3). This is the expected sequence of fixation and agrees with the results of Brown, Fager, and Gaffron (1948) for *Scenedesmus*. After about 60 seconds approximately steady state conditions were attained when fixations in fractions *A*, *B*, and *C* were about 53, 20, and 27 per cent. of the total respectively.

In the dark total fixation of tracer was about one-third of that in the light and did not increase linearly with time (Fig. 1). The reason for this might be that uptake of bicarbonate ion by diatoms in this particular physiological condition is relatively slow in the dark. A rather greater proportion of the total activity was found in fraction *A* but the distribution of tracer was not otherwise greatly different from that found in the light. The statistical significance of the readings made after 30 and 60 seconds exposure is insufficient to show changes in distribution of tracer among the three fractions. For all fractions fixation in the light exceeded by several-fold that in the dark,

showing that the appearance of tracer in these fractions was not due merely to exchange reactions.

A similar experiment was carried out using nitrogen-deficient cells. These were obtained by resuspending cells from a 12-day-old culture in nitrogen-free medium and incubating for a further 24 hours under the usual conditions of culture. For the experiment the cells were resuspended in fresh nitrogen-free medium. The rate of total fixation per unit dry weight of cell material by these cells was about one-third of that of the actively growing cells (Fig. 2).

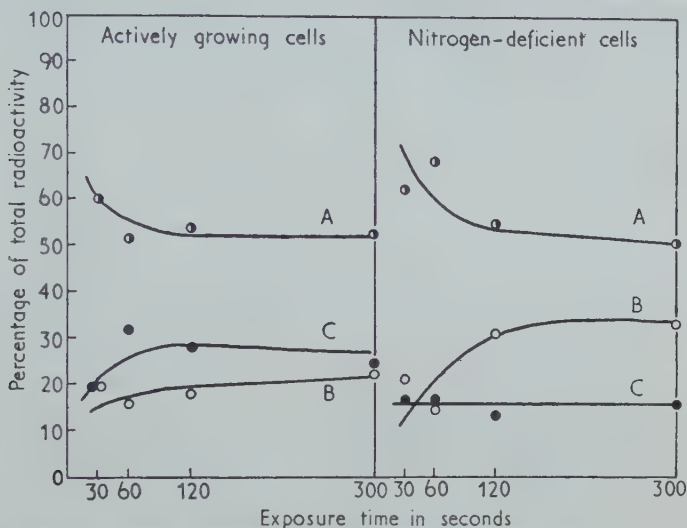


FIG. 3. Changes with time in the distribution among three cell fractions of C^{14} supplied as bicarbonate to actively growing and nitrogen-deficient cells of *Navicula pelliculosa* in the light.

See the legends to Figs. 1 and 2 for details of the material. Radioactivity of the fractions expressed as percentages of the total. Symbols as in Fig. 1.

Again, fixation in the light increased linearly with time, and fixation in fraction A was proportionately greatest for the shorter exposure periods. For the longer exposure periods the proportion of the total activity contained in fraction A was about the same as in the experiment with actively growing cells (Fig. 2). The relative fixations in fractions B and C were, however, the reverse of those found in actively growing cells, that in B being about 33 per cent. and that in C being about 15 per cent.

Total fixation in the dark was again about a third of that in the light. The rate of fixation decreased with time, perhaps as a result of near-saturation of those substances capable of participating in exchange reactions with carbon dioxide. Fixation in fraction A was again higher than in the light, about 70 per cent. of the total, while, relatively to each other, fixations in fractions B and C appeared similar to those in the light.

These two experiments confirmed the expectation that appreciable differences in the pattern of distribution of assimilated carbon into various cell

fractions may exist between cells of the same species in different physiological states. They also showed that, in the light and under the experimental conditions used, fixation of tracer is sufficiently linear with time so that in further experiments it could be assumed that determination of activities after 120 seconds exposure to the tracer solution gave a reasonably accurate measure of steady-state rates of fixation into the three cell fractions studied.

The effect of light intensity

Cells from a 10-day-old culture were resuspended in nitrogen-free medium and samples exposed to tracer in the dark and in light of intensities up to

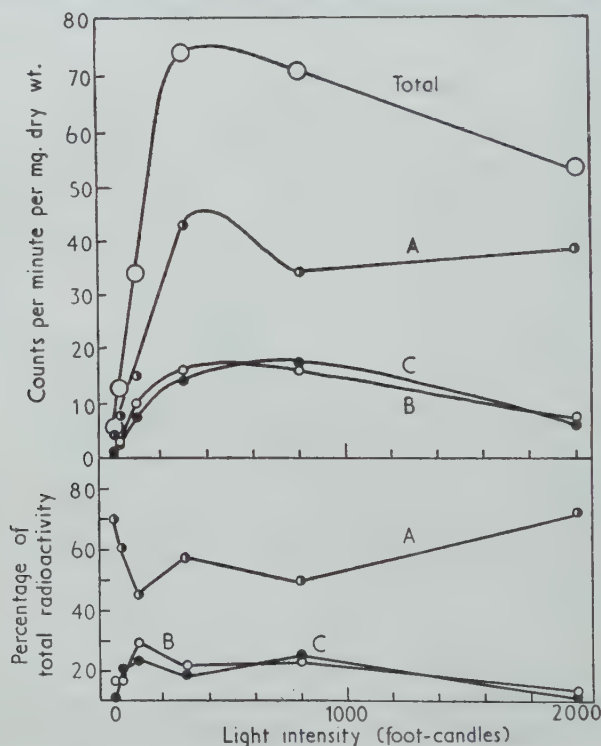


FIG. 4. Effect of light intensity on distribution of radioactivity in cell fractions of *Navicula pelliculosa* exposed to C^{14} -labelled bicarbonate for 120 seconds.

Suspensions with 0.035 ml. cells/ml. and 2.4 mg. dry wt./ml.; N content of cells 3.55% of dry wt.; Medium 'nitrogen-free'. Above: radioactivity as counts/minute/mg. dry wt. of cells; below: radioactivity of the fractions expressed as percentages of the total. Symbols as in Fig. 1.

2,000 foot-candles for periods of 120 seconds. Light intensities were measured with a General Electric light meter. As measured by the total fixation, the rate of photosynthesis increased linearly with light intensity up to about 200 foot-candles, saturation was reached around 400 foot-candles, and at higher intensities the rate diminished, falling to about 70 per cent. of its maximum value at 2,000 foot-candles (Fig. 4). This inhibition at high light intensities

does not appear to result from progressive damage by photo-oxidation since, in other experiments, the rate has been observed to remain nearly constant and growth has occurred during exposures of 4 hours at the highest intensity (see Table II, for example). Inhibition of diatom photosynthesis in the high light intensities obtaining near the surface in the sea and in lakes has often been observed (e.g. by Jenkins, 1937) and may be of a similar nature. Pirson and Wilhelmi (1950) concluded that inhibition of photosynthesis in *Ankistrodesmus* due to nitrogen-deficiency appeared only at high light intensities. It is not known whether the inhibition in *Navicula* is similarly dependent on nitrogen level. The nitrogen content of the cells used in this particular experiment was not very low (3.55 per cent. of the dry weight).

The distribution of the tracer among the three cell fractions varied according to the light intensity. With increasing intensity the relative activity of fraction *A* decreased from its dark value of 70 per cent. of the total to 46 per cent. at 100 foot-candles. Relative fixations in fractions *B* and *C* rose correspondingly and in a parallel manner. Between 300 and 1,000 foot-candles relative fixations in the three fractions remained roughly at the same levels with the proportion of fixation in *A* at about 55 per cent. The depression in absolute fixation of tracer in fraction *A* which occurred at 800 foot-candles is greater than can be accounted for in terms of counting error and was confirmed in replicate analyses. At 2,000 foot-candles fixation in fraction *A* remained at nearly the same level as at 800 foot-candles but fixation in fractions *B* and *C* was less than half that at the lower intensity. The inhibition in the total fixation observed at the highest intensity is almost entirely accounted for by this decrease in fixation in these two fractions.

This finding that fixation in fraction *B*, i.e. fat synthesis (see p. 282), is inhibited at high light intensities is in apparent contradiction to the results of other workers. In an investigation of the variation in the distribution among various substances of radioactive carbon fixed in photosynthesis by *Scenedesmus* it was found that the percentage of the total radioactivity appearing in fats rose as light intensity was increased from 4,000 to 8,000 foot-candles (Calvin *et al.*, 1951). Spoehr and Milner (1949) found that high light intensities favour fat accumulation in *Chlorella*. These differences may well arise from specific characteristics of the algae used or particular experimental conditions but it may be pointed out that in cultures of the type used by Spoehr and Milner the average light intensity reaching individual cells would be only a small fraction of that incident so that, effectively, it may have been about that found optimum for fat accumulation in the experiment just described.

These results show that the standard light intensity selected for the other experiments, i.e. 2,000 foot-candles, was not optimum either for gross photosynthesis or for fixation in fractions *B* and *C* of *Navicula* but there does not appear to be reason for supposing that this invalidates any of the conclusions to be drawn.

The immediate effects on actively growing cells of transfer to conditions of nitrogen-deficiency

The observations on actively growing cells described above were made with suspensions in a medium containing nitrate. Using the same material, parallel observations on tracer fixation during a 120-second exposure were made with a nitrogen-free suspending medium. The cells were held in the nitrogen-free medium for a period of about 45 minutes between harvesting and exposure to tracer.

Lack of nitrate had no great effect on either the total fixation or the distribution of tracer whether the experiment was made in the light or in the dark (Table I). Fixation in fraction *B* in the light is decreased, fixation in fraction

TABLE I

Effect of Nitrogen Supply on the Distribution of Radioactivity in Cell Fractions of actively growing Navicula pelliculosa exposed to C¹⁴-labelled Bicarbonate for 120 seconds

Suspension with 0.0022 ml. cells/ml. and 0.31 mg. dry wt./ml.; N content of cells, 6.4% of dry wt.; N supplied as 0.25×10^{-3} M $\text{Ca}(\text{NO}_3)_2$. Results expressed as counts/min./mg. dry wt. of cells. Figures in parenthesis are percentages of the total radioactivity.

Fraction	With nitrate		Without nitrate	
	light	dark	light	dark
A	132 ± 4.5 (54)	34 ± 3.1 (67)	130 ± 3.3 (56.5)	57 ± 3.5 (82)
B	43 ± 3.2 (18)	2.5 ± 2.5 (5)	27 ± 2.5 (11.5)	10 ± 2.7 (15)
C	69 ± 2.5 (28)	14 ± 1.5 (28)	74 ± 2.5 (32)	2 ± 1.3 (3)
Total	244	50.5	231	69

A in the dark is increased, fixation in fraction *C* in the dark is decreased, in the absence of nitrate. These differences are greater than can be accounted for in terms of counting error and have been found in other experiments.

The immediate effects on nitrogen-deficient cells of transfer to medium containing a nitrogen source

The effect of transferring nitrogen-deficient cells to medium containing ammonium nitrate (Table II) was determined using cells from a 15-day-old culture which had been resuspended and incubated for 48 hours in nitrogen-free medium before being suspended in fresh medium for exposure to tracer.

The supply of ammonium nitrate caused within 5 minutes a statistically significant rise of 12.5 per cent. in the total rate of fixation. Pirson and Wilhelmi (1950) have observed increases in rate of photosynthesis of the same order following supply of nitrate to nitrogen-deficient *Ankistrodesmus*. After 230 minutes' illumination in the presence of ammonium nitrate an increase in the total dry weight of diatom of 35 per cent. was found and the

rate of fixation per unit dry weight had fallen to somewhat below its original value in the nitrogen-deficient material. As a result of the preferential absorption of ammonium ions the pH of this suspension was 7.9, as compared with 8.7 in those subjected to illumination for the shorter period but it seems unlikely that this relatively small change had any great effect on either total fixation or the distribution of tracer among the three fractions (see Ouellet and Benson, 1952, Fig. 1). The changes in the distribution of tracer are more

TABLE II

Effect of Nitrogen Supplied as Ammonia on the Distribution of Radioactivity in Cell Fractions of Nitrogen-deficient Navicula pelliculosa exposed to C¹⁴-labelled Bicarbonate for 120 seconds

Suspensions initially with 0.050 ml. cells/ml. and 1.9 mg. dry wt./ml., rising to 0.055 ml. cells/ml. and 2.6 mg. dry wt./ml. after 230 mins. preillumination. N supplied as 0.004 M NH_4NO_3 at the beginning of the pretreatment period. Results expressed as counts/min./mg. dry wt. of cells. Figures in parenthesis are percentages of the total radioactivity.

Pretreatment time (mins.)	Control, no N		With ammonium nitrate			
	light	dark	light	dark	light	dark
	5	5	5	5	230	230
Fraction A	36.6 ± 4.0 (24)	32.4 ± 3.7 (62)	148.5 ± 5.5 (87)	55.2 ± 4.0 (97)	160.0 ± 5.5 (85)	53.4 ± 4.1 (93.5)
B	106.3 ± 4.9 (70)	14.8 ± 3.6 (28)	12.1 ± 3.3 (7)	0.6 ± 3.2 (1)	12.7 ± 3.5 (7.5)	0.9 ± 0.4 (1.5)
C	8.5 ± 1.0 (6)	5.2 ± 1.1 (10)	9.8 ± 1.3 (6)	1.2 ± 1.0 (2)	14.5 ± 1.3 (7.5)	2.7 ± 1.1 (5)
Total	151.4	52.4	170.4	57.0	187.2	57.0

striking, for, whereas in the nitrogen-deficient cells 70 per cent. of the total radioactivity was found in fraction B, following illumination in the presence of ammonium nitrate for 5 minutes only 7 per cent. was found in this fraction and that in fraction A rose to 87 per cent. This new pattern of distribution was maintained after 230 minutes' illumination in the presence of the nitrogen source. Little change was found in the proportion of tracer entering fraction C. The rate of fixation in the dark was scarcely altered by the supply of ammonium nitrate but changes in the distribution of tracer similar to those found in the light were observed. The dark fixation of carbon dioxide evidently does not reflect the increase in respiration which from the work of Syrett (1953a) one might expect to occur when ammonia is supplied to nitrogen-deficient cells but the increase in fixation in fraction A brought about by the supply of ammonium nitrate both in the light and in the dark may be attributed to the formation of soluble nitrogenous compounds such as Syrett (1953b) has observed in *Chlorella* under similar circumstances. This increased synthesis of soluble materials evidently occurred at the expense of photosynthetic intermediates that otherwise would have been incorporated in fraction B. After 230 minutes slightly increased fixation in fraction C perhaps indicated the beginning of synthesis of these soluble nitrogenous substances to protein.

A corresponding set of observations was obtained with nitrogen-deficient

Navicula supplied with potassium nitrate. These showed no considerable changes within a period of 240 minutes, either in the total fixation or distribution of tracer. Since nitrate must be reduced to ammonia before it can participate directly in metabolism it would be expected that its effect would be much less profound than that of ammonia itself. As with actively growing cells, dark fixation in fractions *A* and *B* was lower, by about 50 per cent., in the presence of nitrate than it was in its absence. This lowering may be accounted for in terms of a decrease in concentration of respiratory intermediates following their utilization as hydrogen donors in nitrate reduction.

The long-term effects on nitrogen-deficient cells of transfer to a medium containing nitrate

Cells from a 9-day-old culture were resuspended in nitrogen-free medium and incubated for a further 24 hours. At the end of this period they were resuspended in 100 ml. fresh nitrogen-free medium. 20 ml. of this suspen-

TABLE III

Growth and Composition of Cells in a Culture Inoculated with Nitrogen-deficient Navicula pelliculosa

Initial culture volume 800 ml. containing 9.8 mg. N supplied as $\text{Ca}(\text{NO}_3)_2$. See text and Fig. 5 for further details.

Day	0	1	2	3
Dry wt. of diatoms (mg./ml. medium)	0.11	0.19	0.25	0.275
Packed-cell volume (ml./ml. medium)	0.0007	0.0017	0.0030	0.0045
N content of cells (% dry wt.)	2.36	4.40	3.49	3.54
Fraction B (% dry wt.)	25	35	27.6	25

sion was used immediately for an experiment in which the distribution of radioactivity in the three cell fractions was determined after 120 seconds' exposure to labelled bicarbonate under the usual conditions. The remaining portion was used as inoculum for a culture in 720 ml. of medium containing nitrate (but no soil extract) which was then incubated under the usual conditions. At intervals of 24 hours 200 ml. portions of this culture were removed and the cells resuspended in nitrogen-free medium and used in further tracer experiments.

Growth, measured as packed cell volume or dry weight per unit volume of medium, occurred throughout the period of observation (Table III). The slowing down observed after the first day cannot be accounted for by exhaustion of nitrate since this was still present in the medium at the end of the experiment. The nitrogen content of the cells almost doubled during the first day but thereafter declined. Contrary to expectation, the fat content of the cells also rose during the first 24 hours following supply of nitrate and at the end of the experiment was still comparatively high.

The results of the tracer experiments carried out on the samples from this

culture are shown in Fig. 5. The rate of total fixation per unit dry weight of algal material was more than doubled within the first 24 hours of culture but fell after this to about its original value. Since the experimental system used was not a closed one, these rates of tracer fixation cannot be used for the accurate calculation of increase in dry weight due to photosynthesis. Nevertheless, as Table IV shows, if it is assumed that no appreciable quantity

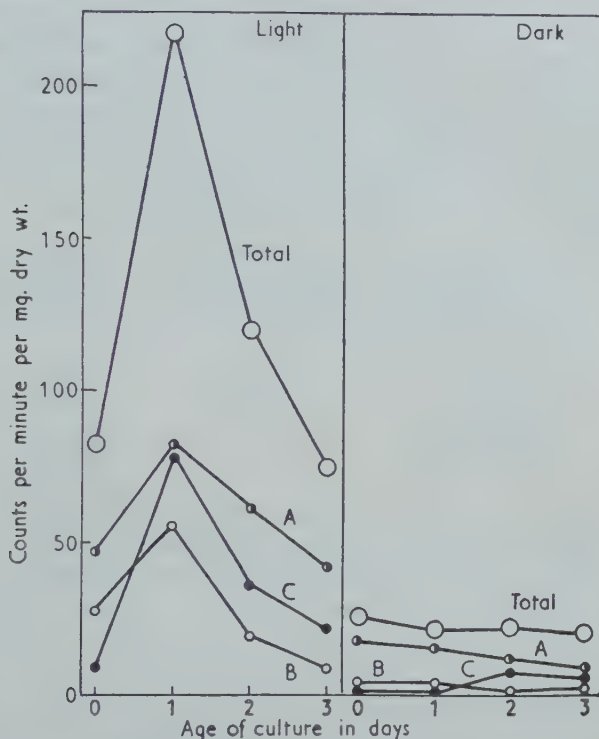


FIG. 5. Distribution of radioactivity in cell fractions of *Navicula pelliculosa* taken at intervals from a culture of nitrogen-deficient cells inoculated into fresh nitrate-containing medium and exposed to C^{14} -labelled bicarbonate for 120 seconds in the light and in the dark.

See Tables III and IV for details of the material. Radio-activity expressed as counts/min./mg. dry wt. of cells. Symbols as in Fig. 1.

of tracer was carried away from the suspension by the air bubbled through it, there is reasonable agreement between calculated and observed increases in dry weight in the culture. The discrepancy for the first 24-hour period suggests that the maximum rate of photosynthesis achieved exceeded that observed in the sample taken after 24 hours.

Fixation in all three fractions increased following the supply of nitrate but the increase in fraction C was relatively greatest, increasing from 12 to 36 per cent. of the total. In the first 24 hours the proportion of fixation in fraction B remained about the same while that in A fell from 59 per cent. to 38 per cent. In the subsequent 48 hours relative fixation in fraction C decreased only slightly but that in A rose to a level approaching that in the

original nitrogen-deficient cells, relative fixation in fraction *B* showing a corresponding decrease. Qualitatively, these changes correspond with the observed changes in cell composition. Thus the increase in relative fixation in fraction *C* and rise in cell nitrogen content in the first 24 hours can both be taken as evidence of increased protein synthesis. The maintenance of a

TABLE IV

Observed Increases in Dry Weight in a Culture of Navicula pelliculosa compared with Increases Calculated as Glucose from Rates of Fixation of Tracer Carbon

Total excess base in experimental suspension = 0.80 mg. Na, pH 8.7, solution in equilibrium with CO₂ at 0.04 atm.; 1 mg. CO₂ (all forms) ~ 432,500 counts. It is assumed that 15% of the dry wt. of the diatom is ash and that the rate of photosynthesis in the culture was 1.27 times that in the tracer experiment (correction for difference in light intensity, see Fig. 4). The rate of tracer fixation taken is the net rate (light-dark value).

Day	Tracer fixation (counts/min./mg./ 120 secs. exposure)	Amount of diatom (geometric mean mg. dry wt./ml.)	Organic matter synthesized (mg.)	
			Calc.	Obs.
0-1	126.0 (mean)	} 0.144	0.026	} 0.068
	197.4 (final)		0.041	
1-2	147.8 (mean)	0.218	0.046	0.051
2-3	76.85 (mean)	} 0.262	0.029	} 0.021
	55.5 (final)		0.021	

high level of fixation in fraction *B* for the first 24 hours followed by a steady decline correlates with the observed initial increase followed by a decrease in the proportion of this fraction in the cells. A quantitative correlation between rates of fixation and amounts cannot be attempted in the absence of information about rates of turn-over of materials in these fractions.

The total dark fixation of tracer per unit dry weight of cell material was scarcely altered following the supply of nitrate and shows no correlation with photosynthetic activity. Changes in the distribution of tracer among the fractions were broadly similar to those observed in the light but seem to have occurred 24 hours later.

The long-term effects on actively growing cells of transfer to conditions of nitrogen deficiency

Cells from two 7-day-old cultures were resuspended in fresh medium (with nitrate but without soil extract), incubated for a further 48 hours under the usual conditions, then washed and resuspended in 700 ml. of nitrogen-free medium. The cells in 100 ml. of this suspension were resuspended immediately in 25 ml. of fresh nitrogen-free medium and used in a tracer experiment of the same type as that just described. The remainder was incubated under the usual conditions and cells from 100 ml. samples, taken at intervals up to 7 days, were resuspended in 25 ml. fresh nitrogen-free medium and used for similar tracer experiments.

There was slow increase both in the packed-cell volume and dry weight

in this culture during the first 5 days (Table V). Packed-cell volume continued to increase to the end of the experiment but the dry weight was found to be distinctly less on the seventh than on the fourth day. This may be ascribed to senescence resulting in hydrolysis and loss of soluble substances from the cells to the medium. Nitrogen content on a dry-weight basis decreased with time. The proportion which fraction *B* formed of the total dry weight decreased somewhat during the first 2 days then rose to almost twice its lowest value. This final rise, however, was due more to decrease in the amount of other cell components than to increased synthesis of fatty materials. The amount of fraction *B* per unit volume of cell material remained nearly constant throughout the period of observation.

TABLE V

Growth and Composition of Cells in a Culture in Nitrogen-free Medium Inoculated with actively growing Navicula pelliculosa

Initial culture volume 700 ml. See text and Figs. 6 and 7 for further details.

Day	0	1	2	3	4	7
Dry weight of diatoms (mg./ml. medium)	0.77	0.87	0.90	0.93	1.05	0.92
Packed-cell volume (ml./ml. medium)	0.011	0.012	0.013	0.014	0.016	0.020
N content of cells (% dry wt.)	3.0	2.7	2.6	2.5	2.2	—
Fraction B (% dry wt.)	19.8	17.0	16.7	21.5	19.7	32.5
Fraction B (mg./ml. cells) . . .	13.9	12.3	11.2	14.5	12.9	14.5

The results of the corresponding tracer experiments are shown in Figs. 6 and 7. Total fixation of tracer in the light (Fig. 6) fell to about a third of its initial value in 3 days. A reduction in rate of photosynthesis of this magnitude when algal cells become nitrogen deficient has been recorded several times (e.g. by van Hille, 1938; Pirson and Wilhelmi, 1950). However, on the fourth day there was a rise in total tracer fixation, considerably greater than can be accounted for in terms of counting error and sustained until the end of the experiment. This rise is entirely accounted for by a rise in rate of fixation in fraction *A*, which after falling until the third day became nearly trebled on the fourth day. Rate of fixation in fraction *B* fell throughout the experiment except for a statistically significant rise on the third day, when fixation in this fraction accounted for 52 per cent. of the total. This corresponds with the beginning of a rise in the proportion of fraction *B* in the cell material (Table V). The final rate of fixation into fraction *B* was only a half of the maximum. Rate of fixation into fraction *C* fell from the second day onwards.

Dark fixation in all three fractions decreased throughout the experiment (Fig. 7) except for that in fraction *A* which rose abruptly, by a statistically significant amount, on the third day. The curve for dark fixation is strikingly similar in form to that for the respiration of ripening fruits (e.g. Fig. 25,

James, 1953) and, no doubt, the processes involved are essentially similar. The rise in fixation into fraction *A* is probably related to the increased tendency to hydrolysis in the cells. Pearsall and Loose (1937) found that in ageing cultures the concentrations within the cells of *Chlorella* of constituents such as hexoses, disaccharides, and soluble nitrogenous compounds rose abruptly. A similar increase of respiratory substrates within *Navicula* cells

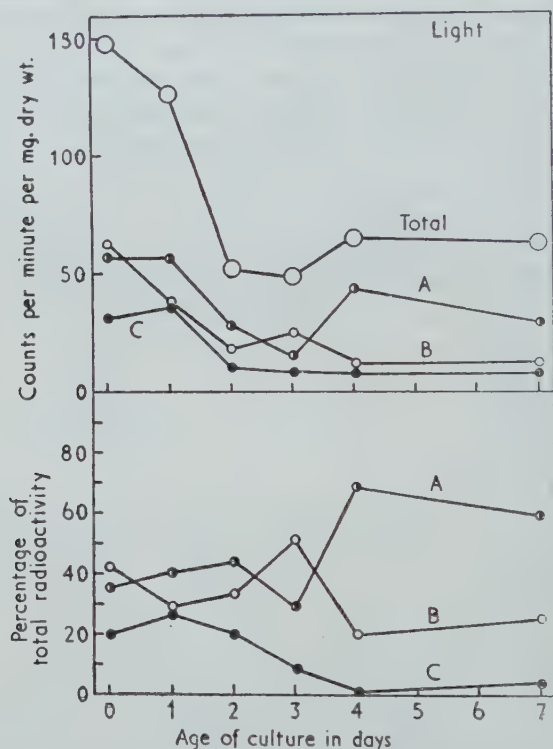


FIG. 6. Distribution of radioactivity in cell fractions of *Navicula pelliculosa* taken at intervals from a culture of actively growing cells inoculated into 'nitrogen-free' medium and exposed to C^{14} -labelled bicarbonate for 120 seconds in the light.

See Table V for details of the material and Fig. 7 for the corresponding results for dark fixation. Above: radioactivity expressed as counts/minute/mg. dry wt. of cells; below: radioactivity of the fractions expressed as percentages of the total. Symbols as in Fig. 1.

becoming increasingly nitrogen deficient would lead to increased concentrations of intermediates susceptible to carboxylation and hence to an increase in fixation in fraction *A*. The rise in fixation in the light on the fourth day would thus appear to be due not to an increase in photosynthesis but to an increase in the size of the pool of substances capable of exchange reactions with carbon dioxide. It will be noted that the increases in the light and dark are of the same magnitude and in the same fraction. That the rise in the light follows that in the dark 24 hours later can be ascribed to the process of breakdown having not proceeded so far by the third day that it cannot be reversed by supply of energy.

Similar results were obtained in another experiment carried out in the same way with material from an 11-day old culture incubated in fresh medium containing nitrate for 48 hours. It would seem from these two experiments that the critical cell nitrogen content below which dark fixation of carbon dioxide in *Navicula pelliculosa* shows an abrupt rise is between 2.5 and 2.0 per cent. on a dry-weight basis, under the experimental conditions used.

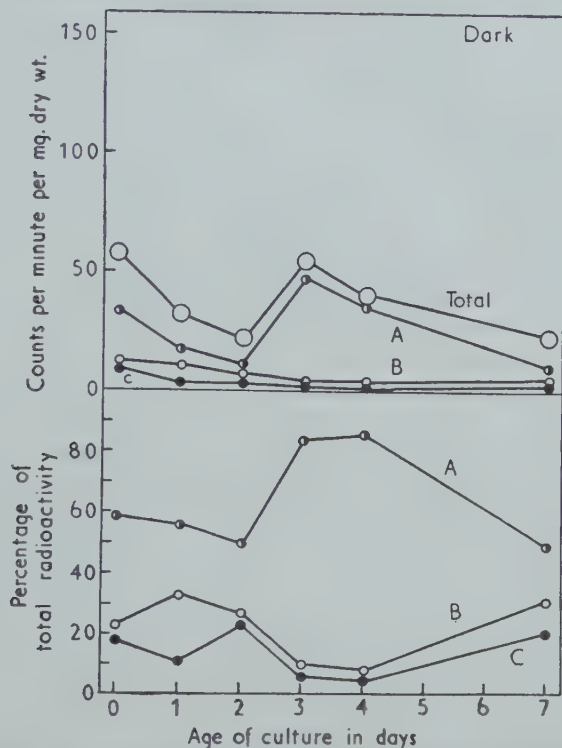


FIG. 7. Distribution of radioactivity in cell fractions of *Navicula pelliculosa* taken at intervals from a culture of actively growing cells inoculated into 'nitrogen-free' medium and exposed to C^{14} -labelled bicarbonate for 120 seconds in the dark.

See Table V for details of the material and Fig. 6 for the corresponding results for light fixation. Above: radioactivity expressed as counts/minute/mg. dry wt. of cells; below: radioactivity of the fractions expressed as percentages of the total. Symbols as in Fig. 1.

DISCUSSION

The results of this investigation confirm that carbon fixed in photosynthesis is rapidly incorporated in substantial amounts into insoluble and fatty cell constituents as well as into sugars and the intermediates involved in their synthesis, and are consistent with the idea that photosynthetic carbon assimilation is closely intermeshed with other metabolic processes through common intermediates. No detailed information about the mechanism of synthesis of fats or proteins from the primary products of photosynthesis has been obtained, however. It remains to be seen, for example, whether the

hydrogen donors necessary for the synthesis of fatty acids are derived directly from the photochemical process or whether they are supplied indirectly via the usual respiratory mechanisms.

The expectation, that the proportions of assimilated carbon following the various anabolic pathways should vary according to the physiological condition of the organism and the conditions to which it is exposed, has also been substantiated. The magnitude of the variation encountered may perhaps best be expressed in terms of the ratio of the rates of fixation in fractions *B* (soluble in 80 per cent. ethanol and in benzene) and *C* (insoluble), the extreme values found being 12.6 and 0.44. This parallels the striking variation in the cell composition of *Chlorella* found by Spoehr and Milner (1949). In rapidly growing cells having a high nitrogen content a large proportion of the carbon assimilated enters fraction *C*, which contains the proteins besides other cell components insoluble in 80 per cent. ethanol. In cells which have ceased growth and which have a low cell-nitrogen content, a major portion of the carbon fixed is incorporated in fraction *B* in correspondence with the accumulation of fats which occurs in such cells. The proportion of carbon assimilated into fraction *A* (soluble in 80 per cent. ethanol, insoluble in benzene) also varies considerably according to circumstances but this proportion would be expected to have little direct relationship with the total amount of any particular cell component since the fraction includes a variety of labile intermediates present in limited amounts only. It is probable that experiments of longer duration would show early saturation of this fraction with tracer.

In discussing the mechanism of these changes in distribution of assimilated carbon, attention will be concentrated mainly on those involving fraction *B*. Presumably, similar considerations apply, *mutatis mutandis*, to the assimilation of carbon into fraction *C*. It will be assumed that the principal components of fraction *B* and those in which tracer is mainly incorporated, are fatty acids. In justification of this assumption it may be said that Collyer and Fogg (1955) found about 80 per cent. of this fraction of *Navicula pelliculosa* to consist of fatty acids and that Clendenning (1950) found that tracer carbon in this fraction of *Scenedesmus* after short periods of photosynthesis with labelled bicarbonate was distributed uniformly among saturated and unsaturated fatty acids and water-soluble saponification products but was absent from the chlorophylls.

In a culture of *Navicula pelliculosa* in a limited volume of culture medium the proportion of fatty acids in the total algal material increases steadily with age, this increasing tendency to fat synthesis being associated with decreasing cell-nitrogen content (Collyer and Fogg, 1955). A similar relationship is evident in the results of the present investigation, for when the percentage of the total radioactivity fixed in fraction *B* in the various experiments is plotted against cell-nitrogen content the line of best fit to the points is one having a negative regression coefficient. However, the points show considerable scatter, the difference of the regression coefficient from zero being barely

significant statistically ($P \sim 0.05$), and it is evident that the relationship is not a straightforward one.

It seems that this change with age in the proportion of assimilated carbon synthesized to fatty acids is primarily dependent on a change within the cells, in enzymic makeup, for example, rather than solely upon concentrations of substrates as was implied in Beijerinck's (1904) original hypothesis concerning fat accumulation. Evidence for this is provided by the considerable stability of a particular pattern of tracer distribution in the face of change in environmental conditions. Thus nitrogen-deficient cells supplied with nitrate continue to synthesize fatty acids at a high rate for as long as 24 hours afterwards whereas other cell properties, e.g. rate of photosynthesis, show considerable response to the altered conditions within this period. Subsequently the capacity to synthesize fatty acids may diminish although the cell-nitrogen content is falling (Fig. 5). This leads to a reversal of the relationship between fatty-acid content and nitrogen content typical of cultures in a limited volume of medium to which no addition is made. Conversely, when actively growing cells are transferred to a nitrogen-deficient medium the proportion of assimilated carbon entering the fat fraction may fall at first and show an increase only after several days (Fig. 6). These findings support the conclusion (Collyer and Fogg, 1955) that the concentration of the nitrogen supply has no direct effect on fat accumulation. It may be postulated that the responses to changed supply of nitrogen are slow because they depend on the enzymic balance of the cells and this may only be changed by synthesis of fresh enzymes during growth or differential inactivation during protein degradation.

Nevertheless, it is an over-simplification to suppose that fat synthesis depends only on the relative concentrations of the enzymes concerned. Were this the case, a close correlation would be expected between carbon fixation into fatty acids in the light and in the dark. There is, in fact, a positive correlation between the percentages of the total radioactivity found in fraction *B* in the light and the dark in the various experiments but it is not statistically significant ($r = +0.38$, $N = 15$, $P > 0.1$) and there are obviously other factors to be taken into account. The variation in the proportion of carbon assimilated to fat at different light intensities seems to depend on two different factors. As light is increased from zero to about 100 foot-candles, substrate concentration appears to be the determining factor. As the concentration of primary products of photosynthesis rises, a greater proportion can be used for synthesis. The decrease in fat synthesis evident above 1,000 foot-candles might be due to the accumulation of a substance in inhibitory concentrations or to inhibition of some enzyme but appears to be something affecting fat and protein synthesis equally. It may be noted here that the effect of light intensity on the distribution of assimilated carbon observed in this work may explain the discrepancy between the recorded values for photosynthetic quotients of algae, which generally approximate to unity and therefore indicate the predominance of carbohydrate synthesis, and the nature

of the reserve products, which for certain of the species involved appear to be fats rather than carbohydrates (Fogg, 1953, p. 112). Apart from the fact that the material used for these determinations of photosynthetic quotient may have consisted of actively growing cells transferred to a medium unsuitable for growth, it seems likely that at the high light intensities which are customarily employed in making the determinations, fat synthesis may have been inhibited so that the values observed correspond with the formation of substances included in fraction *A* in this investigation and having an average composition approximating to that of carbohydrate. To observe photosynthetic quotients corresponding to the formation of fat, it would appear to be necessary to use material from a nitrogen-deficient culture and to expose it to a moderate light intensity.

When ammonium nitrogen is supplied to cells of *Navicula* the effect of substrate concentration on the distribution of assimilated carbon becomes overwhelming. Whereas nitrate evidently does not react directly with the intermediates of the metabolic pool, ammonia undergoes direct reaction with certain of these substances to form soluble organic derivatives which become further elaborated to protein by relatively slow processes (Syrett, 1953*b*). So long as free ammonia is present the primary products of photosynthesis will be used for the synthesis of these substances and little will be available for fat synthesis.

While the proportion of fats to other organic constituents may be determined in general by relative rates of synthesis, it is evident that in senescent cells it may increase as a result of the more rapid hydrolysis of other materials and consequent loss by respiration or diffusion from the cells. Perhaps this depends simply on the lipid phase being less accessible to enzyme action than are constituents in the aqueous phase of the protoplasm. This effect, which may perhaps be termed 'fatty degeneration', occurred in the experiments of the present investigation following the falling of cell-nitrogen content below a particular level but might be expected to occur also as a result of deficiencies of other materials (e.g. water), temperature changes, or the presence of toxic substances.

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The Free and Combined Amino-Acids in some Plant Juices

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ABSTRACT

An examination of the amino-acids present in the 75 per cent. alcohol-soluble and insoluble parts of a number of plant juices has been made. Details are given of a technique which enables twenty-two of these to be estimated with an accuracy of 5 per cent. of the amount present, based on paper chromatography and the ninhydrin reaction carried out under strictly standardized conditions.

THE free and combined amino-acid content of the plants *Beta vulgaris*, *Malus sylvestris*, *Funaria hygrometrica*, *Pinus sylvestris*, *Mucor mucedo*, and *Equisetum arvense*, as determined by quantitative paper chromatography, has already been reported briefly by us (Mansford and Raper, 1954). In response to a number of requests we wish to report the method used as this was the outcome of an exhaustive and critical experimental examination (Mansford, 1954) of the technique of quantitative paper chromatography.

EXPERIMENTAL

Washed plant material was dried at room temperature between blotting-paper, ground finely, and extracted with successive small quantities of 75 per cent. ethanol until the extract gave no ninhydrin reaction. The residue 'A' was set aside, and the united extracts centrifuged and evaporated in a vacuum desiccator, dissolved in water (2 ml.), and the nitrogen in half the solution estimated by the micro-Kjeldahl procedure of Chibnall (1943). The remainder was 'desalted' by adsorption on a column of *Permutit* special sulphonated polystyrene resin and elution with N. ammonium hydroxide after washing free from mineral matter, experiment having shown that electrolytic desalting resulted in considerable loss of, e.g., arginine and glutamine. The eluate was evaporated in a vacuum at room temperature and the residue dissolved in such a quantity of water that 1 ml. of solution contained 10 mg. nitrogen. Pre-washed Whatman No. 1 filter paper (chromatography grade) was used, washing being carried out as in downward chromatography, allowing water to run for 24 hours after the front had reached the bottom edge of the paper and drying spontaneously overnight. The recovery of some amino-acids was considerably increased by this washing procedure. Acid washing (Thompson, Zacharius and Steward, 1951) effected no further improvements.

A volume of the solution 'B' containing the desired amount of nitrogen

(40–500 μg . according to the amount of the amino-acids it was desired to estimate on the particular chromatogram) was adjusted to pH 6.2 and applied to the paper within a circle of radius 0.25 cm. whose centre was 5 cm. from each of two adjacent edges of the sheet. The paper was suspended over a glass rod in the chromatography chamber so that approximately equal lengths hung over each side, the edge with the applied amino-acids being just above the surface of the solvent in the bottom of the chamber, and held in position by stainless-steel clips. Neither the rod nor the clips interfered in any way with the running of the chromatogram. The sides of the chamber were lined with filter paper saturated with solvent (McFarren, 1951). The closed chamber was left undisturbed for 24 hours, after which the edge of the paper was lowered so that the applied material was 3 cm. from the surface of the solvent, Burma and Banerjee (1951) having shown that variation in the distance of the point of application of the material from the solvent surface affects R_F values. Since these also vary with temperature (Consden, Gordon and Martin, 1944; Burma, 1951) and higher temperatures cause the amino-acids to spread in the paper, the temperature of the tanks was controlled within 2°C . by lagging, and, if necessary, wrapping with thermostatically controlled electric heating tape. The solvent was allowed to run up the paper, over the rod, and down the other side for a total distance of 35 cm. The first solvent was phenol distilled from zinc dust and equilibrated at 18° with a 0.02 M. phosphate aqueous buffer solution of pH 7.5 (Sørensen, 1909). Several workers (Novellie, 1948; Brush *et al*, 1951; Fowden, 1951a) have shown that if papers containing phenol are dried by heating, marked losses of many amino-acids occur. We therefore suspended the papers after the run and washed the phenol from them with dry acetone applied from an all-glass spray before drying in a current of cold air for 30 minutes. In the absence of water in the acetone this procedure reduced the loss of alanine, for example, from 15 to 3 per cent. even if the paper was subsequently heated at 70° for 2 hours, without affecting the R_F values or causing diffusion of the spots. The paper was then turned through 90° , suspended 24 hours in a chamber containing the second solvent (*n*-butanol shaken with an equal volume of aqueous pyridine prepared by mixing equal volumes of pyridine and pH 7.5 buffer), lowered as before into the solvent, and then allowed to run 35 cm. The solvent was then removed with dry acetone and the paper dried in the same way as before.

For the detection and estimation of the amino-acids we used, after exhaustive trials of other methods, the ninhydrin reaction under standardized conditions. Ultra-violet fluorescence (Phillips, 1948; Woiwod, 1948, 1949, 1950; Fowden, 1951, 1952) was found to be unreliable unless the papers were dried at at least 100° , under which circumstances quantitative recovery of many amino-acids is impossible. Folin's reagent showed no advantage over ninhydrin. The use of an iodine spray (Brante, 1949) gave, like other methods, results which, for our purposes, were neither sufficiently sensitive nor consistent.

The dry chromatogram was sprayed evenly on both sides with 1 per cent. methanolic potassium hydroxide, heated 5 minutes at 60° (this procedure

greatly reduces background colour with the ninhydrin reaction) (Fowden and Penney, 1950), sprayed lightly with 0.05 per cent. methanolic ninhydrin and heated 3 minutes at 60°. The light blue spots served as a guide for cutting up the chromatogram into squares of known area, each of which contained one amino-acid. By strictly observing the above procedure 21 amino-acids can be completely separated. Special difficulties arising in the separation and estimation of others are described below, with the methods adopted to overcome them. The reagent for quantitative colour determination was ninhydrin (4 g.) freshly recrystallized from 2 N. hydrochloric acid, dissolved in peroxide-free methylcellosolve (100 ml.) in an atmosphere of nitrogen. Stannous chloride dihydrate (160 mg.) in 0.2 M. citrate buffer of pH 5.0 (100 ml.) was added, any precipitate which formed overnight removed by filtration in a nitrogen atmosphere, and the reagent stored under nitrogen in dark bottles in the refrigerator (Moore and Stein, 1948). Ammonia must be strictly excluded.

Each cut-out square of the chromatogram containing one amino-acid was placed in a test-tube, ninhydrin reagent (1.5 ml.) added, and the tube closed with an aluminium cap. Three squares of paper of known area from different parts of the sheet of paper known to be free from amino-acids were treated similarly to determine the background colour. Unless these gave consistent results the chromatogram was rejected. The tubes were loaded in a rack, heated in a boiling water bath 25 minutes with constant agitation, and cooled in ice water. 50 per cent. ethanol (10 ml.) was added to each tube, the contents gently shaken to avoid disintegration of the paper, and decanted. The extraction of each square of paper was repeated (50 per cent. ethanol, 10 ml.) and the total volume made up to 25 ml. with 50 per cent. ethanol. The advantages of this method of carrying out the ninhydrin reaction in a test-tube as opposed to the reaction on the paper method of Thompson *et al.* (1951) are as follows:

1. The need for anaerobic conditions which can be fulfilled more easily in a test-tube than on the surface of a large sheet of paper.
2. The reaction conditions of a temperature of 100° C. for a period of 25 minutes can be controlled much more readily with a series of test-tubes.
3. The instability of the colour product on wet paper under aerobic conditions.
4. The difficulty in obtaining complete reaction on the paper.
5. The anomalous behaviour of asparagine when the reaction is carried out on paper (Steward and Thompson, 1952).

The colour density of the various extracts was determined with a Unicam S.P. 600 spectrophotometer at 570 $m\mu$, this being a wavelength of maximum light absorption of the ninhydrin product from amino-acids which behave normally with this reagent. The higher absorption maximum (410 $m\mu$) is less suitable because of the greater background colour. Since the colour gradually fades all readings were taken as soon as possible and in no case more

than 2 hours after the colour development. The amount of amino-acid in each spot was determined from the colour density, the densities corresponding to known weights of amino-acid being independently determined for various known weights of the pure amino-acids run on chromatograms under our standard conditions. The colour-density-weight line for each amino-acid was determined from a number of weights of each acid, each point plotted being the mean of three determinations not differing by more than 5 per cent. of the actual value. The literature dealing with the relation between amino-acid loss during chromatography and distance of movement is contradictory. Woiwod (1948) reported that there was a relation between these factors in the case of glycine, but Fowden and Penney (1950) claimed that glycine is atypical and they were able to show no relation between loss of amino-acid and distance of travel. Our own results (Mansford, 1954) are in agreement with those of Thompson (1951) and led to the use of a constant solvent run in order to obtain strictly reproducible results. We found there was a loss of amino-acid roughly proportionate to the distance travelled, the proportionality factor differing from acid to acid. The loss was greater with phenol than with butanol-pyridine solvent, and for hydroxyamino-acids than for those hydroxyl-free.

In some cases as an additional check a known amount of an amino-acid known to be absent from the plant extract was added to it prior to chromatography, and an empirical factor, acid added/acid recovered, determined. This correction was in no case significant enough to necessitate its use, but corrections for background colour were sufficiently high to warrant careful determination (up to colour density of 0.05 in extreme cases). These factors are particularly important for those acids less sensitive to ninhydrin.

Because of the wide range of concentrations of amino-acids in the various extracts, they could not all be estimated on a single chromatogram, since if the extract containing enough of those present in small amounts was applied to the paper, those present at higher concentrations gave overlapping spots. Several sets of chromatograms were therefore made from each extract, using amounts of amino-acids varying from 50 to 500 μ g. of nitrogen. The results are tabulated below. Every quantitative determination is the result of three consistent chromatograms.

Proline and hydroxyproline cannot be estimated satisfactorily by the ninhydrin reaction. The areas on the chromatograms containing them were detected by their positions relative to the other amino-acids and cut out, together with blanks of similar area from a part of the paper free from amino-acids. Each square was placed in a separate test-tube, heated for 10 minutes in a boiling-water bath with 5 ml. of isatin solution (0.5 per cent. in *n*-butanol containing 5 per cent. V/V of acetic acid), the colour extracted as above, the solution made up to 25 ml. with 50 per cent. ethanol, and the colour densities at 585 $m\mu$ compared with those given by standard amounts of the amino-acids.

Citrulline and β -alanine are not separated by the above procedure. The presence of citrulline was detected by applying to the area of the chromato-

gram where it might be expected with β -alanine a 1 per cent. solution of *p*-dimethylaminobenzaldehyde in N.HCl, with which it gives a yellow colour (Fowden, 1951 *b*). A negative response to this test enabled β -alanine to be estimated in the usual way, but if a positive result was obtained special chromatograms were run for their estimation, using *m*-cresol-aqueous buffer pH 5.7 (Sørensen, 1909) as the second solvent, which enables these acids to be separated.

Lysine and ornithine are confused on the standard chromatograms. They, together with arginine and histidine, can be separated from all other common amino-acids except methionine by one-dimensional chromatography in *m*-cresol-aqueous buffer pH 9. The methionine was converted into its sulphoxide and sulphone (estimated together) by oxidation with hydrogen peroxide (Dent, 1948), which also converts cysteine to cysteic acid (estimated as such). This treatment gives consistent and reliable recoveries of lysine, ornithine, arginine, and histidine from synthetic mixtures, but threonine and serine gave variable results. The sulphur-containing amino-acids were therefore estimated from separate two-dimensional chromatograms in order not to reduce recoveries of the hydroxy-acids.

Phenylalanine, methionine, leucine, and *isoleucine* were separated satisfactorily in a mixture of *n*-butanol-benzyl alcohol (1:1) saturated with 0.067 M. sodium borate-potassium chloride buffer pH 8.4 (McFarren, 1951).

A blue-purple spot appearing consistently in the alcohol-soluble extract from *Funaria hygrometrica* (R_F about 0.60 in phenol and 0.05 in butanol-pyridine) was shown by hydrolysis after elution to be a peptide of alanine and glycine. A yellow spot found on some chromatograms from *Mucor mucedo* in the place normally occupied by dihydroxyphenylalanine gave only phenylalanine on hydrolysis.

The residue 'A' from the alcohol extract of the plant juice was in each case hydrolysed by heating at 100° C. for 24 hours in a sealed tube with a mixture of concentrated hydrochloric acid (2.5 ml.) and glacial acetic acid (2.5 ml.) to which was added stannous chloride dihydrate (0.15 g.) (Fowden, 1952). The amino-acids in the hydrolysate were separated and estimated in the same manner as those in the ethanolic extract.

Treatment of synthetic mixtures of amino-acids in this way gave recoveries of at least 90 per cent. of each acid.

RESULTS

The complete analytical results are given in one instance only (75 per cent. ethanolic extract of the juice of *Mucor mucedo*) (Table I). Tables II and III give the summarized results for other plants, while Tables IV and V give the amino-acid analysis of *Equisetum arvense* at fortnightly intervals during various periods of its growth, the fifth being at spore formation.

DISCUSSION

Many analyses of plant amino-acids have been made, and the results of those effected by paper chromatography are tabulated in the thesis to which

TABLE I

Amino-acids in the 75 per cent. Alcohol-soluble part of the juice of Mucor mucedo

Amino-acid	Micrograms of amino-acid found			Mean	μg. of N found	% of total N
50 μg. N applied (2-dimensional separation)						
γ-aminobutyric acid	25.0	25.0	25.2	25.2	3.42	6.8
Asparagine	23.0	23.5	21.0	22.5	4.77	9.5
Aspartic acid	109.0	110.0	106.0	108.3	11.39	22.8
Glutamine	38.5	40.0	40.0	39.5	7.60	15.2
Glutamic acid	62.0	60.0	61.0	61.0	5.81	11.6
200 μg. N applied (2-dimensional separation)						
Alanine	23.0	23.5	23.0	23.2	3.65	1.8
Glycine	20.5	21.5	19.5	20.5	3.83	1.9
Proline	22.0	22.0	25.0	23.0	2.80	1.4
Serine	37.0	37.0	34.0	36.0	4.80	2.4
Threonine	19.0	18.0	18.0	18.7	2.20	1.1
Tyrosine	30.0	33.0	30.0	31.0	2.40	1.2
Valine	50.0	49.0	48.0	49.0	5.86	2.9
400 μg. N applied after peroxide treatment						
Methionine	25.0	26.0	26.0	25.7	2.40	0.6
200 μg. N applied (1-dimensional separation by m-cresol)						
Arginine	30.0	30.0	30.0	30.0	9.65	4.8
Lysine	37.0	39.0	37.5	38.5	7.38	3.7
200 μg. N applied (1-dimensional separation by benzyl alcohol-butanol)						
isoleucine	36.0	35.0	33.5	35.0	3.79	1.9
Leucine	45.0	45.0	44.5	44.8	4.78	2.4
Phenylalanine	21.0	21.0	21.5	21.2	1.80	0.9
Total	—	—	—	—	—	92.0%

TABLE II

Amino-acids Present in 75 per cent. Alcohol Extracts

Acid	Beta vul-garis	Malus sylve-stris	Funaria hygrometrica	Pinus sylve-stris	Mucor mucedo	Equisetum arvense
Alanine	5.3	1.7	1.7	5.4	1.8	5.8
β-alanine	8.3	1.2	—	2.8	—	—
Arginine	3.5	9.2	9.4	9.4	4.8	9.6
Asparagine	7.6	12.2	1.4	2.4	9.5	10.2
Aspartic acid	4.5	7.4	15.4	2.7	22.8	7.4
γ-amino butyric acid . . .	8.0	9.8	5.3	5.3	6.8	3.5
Citrulline	—	—	6.3	—	—	—
Cystine	0.8	—	2.2	—	—	—
Glutamine	26.2	24.8	2.3	5.3	15.2	15.4
Glutamic acid	6.0	8.9	22.4	43.2	11.6	8.2
Glycine	2.0	1.2	0.5	2.4	1.9	2.5
Histidine	3.0	—	—	—	—	—
isoleucine	1.0	2.6	1.2	1.9	1.9	2.9
Leucine	3.4	8.4	10.4	1.7	2.4	7.8
Lysine	2.2	0.8	7.2	1.4	3.7	4.5
Methionine	—	—	—	1.3	0.6	0.9
Proline	—	1.3	—	—	1.4	—
Phenylalanine	2.0	1.4	0.6	—	0.9	0.9
Serine	4.0	0.9	0.6	0.7	2.4	2.5
Threonine	3.6	1.0	0.8	1.6	1.1	4.1
Tyrosine	2.0	0.9	2.4	0.9	1.2	0.9
Valine	2.3	2.4	2.4	4.9	2.9	6.2
Total	95.7	96.1	92.5	93.3	92.9	93.3

TABLE III

Amino-acids Present in Hydrolysates of Alcohol-insoluble Fractions

Acid	Beta vul- garis	Malus sylve- stris	Funaria hygro- metrica	Pinus sylve- stris	Mucor mucedo	Equiset- um arvense
Alanine	6.8	3.6	6.8	3.6	6.7	4.3
Arginine	14.3	15.8	13.2	15.7	12.2	19.5
Aspartic acid	9.0	10.2	7.8	10.2	16.4	8.2
Cystine	1.1	1.0	1.5	1.2	2.2	—
Glutamic acid	21.2	19.2	18.8	29.2	35.2	14.5
Glycine	4.7	3.8	6.5	3.6	3.8	6.5
Histidine	2.0	2.1	—	4.1	1.6	4.2
Hydroxyproline	—	1.0	—	0.9	—	—
isoleucine	2.2	4.1	2.2	6.1	0.7	4.3
Leucine	10.8	18.9	11.3	14.2	5.2	9.6
Lysine	2.5	2.2	8.4	2.2	3.8	11.2
Methionine	2.5	2.1	0.9	1.2	1.1	1.4
Proline	4.5	3.4	4.2	1.7	4.2	5.1
Phenylalanine	3.1	2.4	5.6	2.4	1.0	4.2
Serine	4.5	3.0	3.4	0.6	3.3	2.8
Threonine	4.0	2.3	4.4	0.6	2.2	3.7
Tryptophane	3.2	2.5	—	2.5	—	—
Tyrosine	2.8	2.1	3.9	2.1	2.1	3.7
Valine	6.2	6.0	7.2	1.9	4.4	2.3
Total	105.4	105.7	106.1	104.0	106.1	105.5

TABLE IV

Amino-acids Expressed as Percentage of total N in the 75 per cent. Alcohol-soluble part of the Juice of Equisetum arvense, at Fortnightly Intervals during its Growth, the Fifth being at Spore Formation

Amino-acid	Batch no.					
	I	II	III	IV	V	VI
Alanine	5.8	2.3	1.7	0.9	1.6	2.4
β -alanine	—	—	—	—	—	—
γ -aminobutyric acid	3.5	4.4	3.9	5.3	4.4	5.0
Arginine	9.6	10.4	8.0	9.0	4.5	5.3
Asparagine	10.2	9.7	17.4	15.2	20.0	16.1
Aspartic acid	7.4	8.2	7.1	6.3	1.3	2.0
Citrulline	—	—	—	—	—	—
Cystine	—	—	—	—	—	—
Glutamic acid	8.2	4.1	2.4	1.4	1.1	2.3
Glutamine	15.4	19.7	21.2	32.1	38.3	25.1
Glycine	2.5	4.0	2.1	0.9	1.0	2.1
Histidine	—	—	—	—	—	—
isoleucine	2.9	3.1	3.0	2.7	3.2	3.5
Leucine	7.8	6.8	6.3	6.0	4.5	7.9
Lysine	4.5	5.6	5.6	4.8	3.2	6.1
Methionine	0.9	2.0	0.9	0.0	0.0	0.9
Phenylalanine	0.9	1.5	1.1	0.9	0.9	0.0
Proline	—	—	—	—	—	—
Serine	2.5	2.6	2.1	1.0	1.1	2.8
Threonine	4.1	3.8	3.1	1.5	0.8	1.0
Tyrosine	0.9	1.5	1.5	0.9	1.0	2.8
Valine	6.2	4.3	7.4	5.2	4.1	5.0
Total	93.3	94.0	94.2	94.1	91.0	90.3

TABLE V

Amino-acids in the Alcohol-insoluble Portions of the Juice of Equisetum arvense at the same times as those recorded in Table IV

Amino-acid	Batch no.					
	I	II	III	IV	V	VI
Alanine	4.3	7.8	6.5	6.3	6.9	7.1
Arginine	19.5	16.8	11.2	9.6	5.3	8.5
Aspartic acid	8.2	9.1	9.6	10.8	9.9	10.7
Cystine	—	Trace	1.4	Trace	—	1.4
Glutamic acid	14.5	12.9	14.3	17.6	21.1	19.8
Glycine	6.5	4.7	5.2	6.6	6.5	6.2
Histidine	4.2	3.7	3.7	4.1	3.7	2.1
Hydroxyproline	—	0.7	—	—	0.7	0.9
isoleucine	4.3	5.3	6.2	5.7	5.7	6.9
Leucine	9.6	11.4	12.2	14.9	13.6	13.1
Lysine	11.2	9.6	7.6	8.2	6.4	5.1
Methionine	1.4	1.9	0.9	1.1	1.4	1.2
Phenylalanine	4.2	4.9	5.7	5.9	6.3	6.7
Proline	5.1	5.3	6.9	4.2	2.0	0.9
Serine	2.8	3.4	2.9	3.1	3.5	3.1
Threonine	3.7	3.4	2.5	2.3	3.0	3.2
Tryptophane	—	—	2.0	0.9	—	0.9
Tyrosine	3.7	2.9	3.1	0.9	0.9	2.0
Valine	2.3	3.2	2.9	4.3	5.4	4.9
Total	105.5	107.0	104.8	104.2	102.4	104.7

reference is made (Mansford, 1954). Most of these are qualitative only and refer to plants of immediate economic value as foodstuffs. We have extended these observations to other groups of plants, and have employed a quantitative procedure which does not require elaborate apparatus. The reasons for each detail of the technique are given in the thesis (Mansford, 1954). There would appear to be a wide field for the application of such a method in the study of amino-acid metabolism and the analysis of protein hydrolysates.

Little correlation exists between the taxonomic position of a plant and its amino-acid content. The relatively large amount of glutamic acid in *Pinus sylvestris* is of note, and it would be of interest to ascertain if other conifers have a similar high content, little detailed information on this important group being available.

Whilst γ -aminobutyric acid is of almost universal occurrence in plants (the main exception appears to be in seeds) it appears to be of more importance in the higher plants, the reverse being the case with lysine. The acid is found only in the 75 per cent. alcohol-soluble fraction of the plant juices examined, and may be the precursor of glutamic acid in higher plants, Schales (1946) having shown that there is only minimal glutamic- α -decarboxylase in, e.g., potato tubers, whereas the occurrence in animal cells of γ -aminobutyric acid accounts for the reverse process there.

Only the higher plants examined here contained β -alanine, although Work (1950) has detected it in *C. diphtheriae*.

The existence of twenty-two amino-acids and amides has thus been

demonstrated, and their quantities determined. Many others have been claimed to occur, and Steward and Thompson (1950) have listed a number of substances of unknown nature which give the ninhydrin reaction on paper chromatograms of amino-acid mixtures. The use of cyanides and ammonia vapour with phenol, and of unbuffered solvents, may, however, well produce such substances as artifacts, e.g., by the action of ammonia, or of cyanides in the presence of reducing agents and keto-acids (Steward and Thompson, 1950). For this reason the addition of all such substances was avoided in this work, and very few spots were found in unexpected places, such spots being shown in two cases to be due to peptides. The occurrence of new amino-acids should clearly be based on more substantial evidence than the appearance of a blue spot in the ninhydrin reaction and confirmation by isolation of the pure compound on ion-exchange resin columns as utilized by Grobbelaar *et al.* (1955) is desirable.

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The Effects of Griseofulvin on Certain Phytopathogenic Fungi

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With plates VIII and IX

ABSTRACT

In vitro and *in vivo* investigations have been carried out into the reaction of actively growing hyphae of *Botrytis* spp. on encountering an environment containing griseofulvin.

In vitro, the reactions noted were slowing of the growth rate, stimulation to branching, abnormal appearance of hyphal protoplasm, and loss of rigidity of the hyphal membrane in the region of the tip. The antibiotic is not translocated within the mycelium.

In vivo, *Botrytis tulipae* was incapable of penetrating cell membranes in the stem of tulips watered with griseofulvin. The antibiotic was shown to delay or to prohibit the penetration of onion epithelium by *B. allii*, depending on concentration. The characteristic curling and stunting of hyphae caused by griseofulvin have both been observed to occur in plant cells.

INTRODUCTION

GRISEOFULVIN, first isolated by Oxford, Raistrick, and Simonart (1939), and 'curling factor', by Brian, Curtis, and Hemming (1946), have been shown to be identical (Grove and McGowan, 1947) (Brian, Curtis, and Hemming, 1949). By reason of its marked fungistatic properties, griseofulvin has been the subject of several investigations that have shown it to possess a number of properties desirable in a substance intended to be effective in controlling plant diseases. Brian (1949) has shown that the antibiotic is stable over a wide pH range, is not affected by autoclaving and is fungistatically active against all groups of fungi except the Oomycetes, yeasts, and Actinomycetes. Brian, Wright, Stubbs, and Way (1951) demonstrated that it was systemic in plants, being taken in by the roots of lettuce and oats and translocated to the leaves and remaining detectable in the plant for some time after its source was removed. These authors also achieved control of *Botrytis cinerea* on lettuce and *Alternaria solani* on tomatoes, but found that griseofulvin is quickly degraded in unsterile soil. This communication gives a preliminary report of a research project designed to study the mode of action of griseofulvin, in relation to the control of plant disease.

I. *IN VITRO* EFFECTS

Because of Brian's (1949) detailed description of the effects of griseofulvin on the germination and the resultant hyphae of *Botrytis allii* when grown on a

medium containing this substance, an investigation has been carried out into the reaction of hyphae, previously grown in a normal environment, on suddenly entering one containing griseofulvin. Such a situation could occur in the event of a fungal spore germinating on a leaf epidermis and meeting on penetration an effective concentration of the antibiotic within the plant cells.

Method. The method used (parallel hanging drop cultures) follows closely that previously described (Aytoun, 1953), except that a Winge micromanipulation cell (Winge, 1935) was used instead of the normal glass rings of a van Tieghem cell, in order to obtain better aeration. For preparing the hanging drops the medium used throughout was 2½ per cent. malt agar, filtered and with pH adjusted to 5.5. The griseofulvin was dissolved in acetone and added to the liquid malt agar at between 45 and 55° C., so as to give an acetone concentration of not more than 4 per cent. in the medium.

The Winge cell, its arms plugged with cotton wool, was fixed to a microscope slide by means of a beeswax and soft paraffin mixture, and 2 ml. of sterile water was added to provide moisture. Two malt agar drops, one containing the required concentration of griseofulvin, the other free from it, were placed on a No. 1 coverslip so that their edges were close together but not touching. The drop not containing the antibiotic was inoculated with a few spores of the fungus and the coverslip was inverted and sealed to the Winge cell with soft paraffin. Control cultures were also set up with one of the hanging drops containing acetone at the same strength as was present in the griseofulvin drops. Each culture was placed in a sterile Petri-dish and incubated.

By this method the fungus became established on the inoculated agar drop and eventually grew across and impinged on the agar containing griseofulvin, where its reactions were observed.

Observations on Botrytis spp. This work has been carried out using *Botrytis* spp. because the genus is responsible for disease in a great variety of plants. Broadly speaking, by this method the reactions of the *Botrytis* spp. tested (*B. cinerea*, *B. allii*, *B. tulipae*, and *B. fabiae*) to griseofulvin are identical and their response to concentration is similar to that described for *B. allii* by Brian (1949).

There are four ways in which the reaction of the fungal hypha to a griseofulvin-containing environment shows itself.

(1) *Decrease in growth-rate.* At concentrations between 0.01 and 0.1 µg./ml. reduction in growth-rate can be detected without curling of the hyphae. Concentrations between 0.1 µg. and 1.0 µg./ml. cause the hyphae to curl in the characteristic manner, and at about the latter concentration stunting of the hyphae can be seen. This stunting increases with greater concentrations of griseofulvin, until at about 10 µg./ml. other severe growth abnormalities appear. These take the form of giant cells, Pl. VIII D (mentioned by Brian, 1949), globular excrescences that do not develop further, a thalloid type of growth resembling in form the fronds of *Fucus* and bursting of the hyphae,

Pl. VII E. There is a difference in reaction to concentration, shown by hyphae growing on the surface of the agar, aerial hyphae, and those growing through the agar. Those growing embedded in the medium show a more severe reaction than those on the surface. Aerial hyphae arising from mycelium growing on distinctly high concentrations of the antibiotic appear normal. If the fungus is first established on a griseofulvin-free medium, it is able to continue growing in concentrations of griseofulvin in which spores would merely germinate and stop growing. This has been observed when a few spores have been accidentally scattered on to the griseofulvin hanging drop during inoculation. The spores germinating in the presence of 10–20 $\mu\text{g./ml.}$ of the antibiotic produced a short germ tube only, whereas hyphae growing across from the other drop have been seen to grow slowly through them.

(2) *Stimulation to branch coupled with loss of direction.* A normal fungal hypha on encountering a new nutritional environment forms branches that direct themselves forwards and outwards in an attempt to grow over the environment, Pl. VIII A. In this sense a fungus may be said to show a directional trend in its growth. On entering an environment containing more than $\approx 1 \mu\text{g./ml.}$ of griseofulvin, it forms more than the normal number of branches, and these do not show any directional trend; frequently growing round towards their point of origin, they may aggregate to form a tight knot, Pl. VIII, B and C. At concentrations between 0.1 and 1.0 $\mu\text{g./ml.}$ hyphae, though showing the typical curling form, in general maintain the normal direction of growth.

(3) *Atypical appearance of hyphal protoplasm.* Healthy fungal hyphae, when viewed through a microscope by transmitted light, appeared in the region of the growing tip to be almost homogeneous in content. The tip region of hyphae showing stunting appeared more granular. Small dark bodies of the order of 1μ in size were frequently seen, singly or in groups of up to 6 or 8. They were seen to be in motion if watched for periods of 30 seconds or more. This movement was random, either as a complete group, or individually, in any direction. Attempts have been made to correlate the presence and position of these granules with the formation of hyphal abnormalities, but so far unsuccessfully. These granules are not obvious in curling hyphae.

(4) *Disturbances in hyphal wall rigidity.* Hyphae growing in contact with griseofulvin tend to be larger and more uneven in diameter than in controls, whose diameters are remarkably constant. The abnormalities that appear with the higher concentrations of griseofulvin may be swellings or bulbous excrescences on one side or involving the whole of the hypha, Pl. VIII D. They are formed initially close to the growing tip and have not so far been observed forming farther back than the first septum from the tip. Burst hyphae are frequently seen, Pl. VIII, E, the bursts occurring in the region of the growing tip or, rarely, at the junction of a branch. The quantity of protoplasm expelled on bursting suggests that an appreciable pressure existed within the hypha before rupture.

Discussion. As a result of these *in vitro* observations, it is possible to draw certain conclusions as to the way griseofulvin affects the hyphae of species examined. In the first place the fungus in the form of the young germ-tube is the most severely affected by the antibiotic. The actively growing hypha is able to maintain a restricted degree of growth in concentrations of the antibiotic that would prohibit spores from developing beyond the germ-tube stage. Secondly, to judge from the normal form of aerial hyphae produced from mycelium in contact with and severely affected by relatively high concentrations of the antibiotic, it is clear that griseofulvin is not translocated within the mycelium (suggested by Brian, 1949). It is possible to go farther than this and say that the effect of a given concentration of griseofulvin on a hypha is proportional to the area of the hypha at the growing point in contact with it. Individual hyphae change their degree of reaction to the antibiotic on growing into the agar medium after growing on the surface, and vice versa. Finally, the antibiotic appears to exert its effect on the hypha in the region of the tip. Initiation of swellings and other deformities almost always takes place at a point just behind the tip.

This effect on the hypha in the region of the growing point may be caused in either of two ways. Either the antibiotic influences the formation of the hyphal wall in such a way that extension is inhibited, thereby causing a build up of pressure inside the hypha, which results in swelling or bursting, or the presence of the antibiotic causes abnormally weak areas in the wall, which are unable to withstand the pressures of normal turgor. At the present stage of the work it is impossible to decide between these two explanations, but it is hoped to study this problem in the near future.

II. INVESTIGATIONS *IN VIVO*

Introduction. An attempt was made in these experiments to obtain control of tulip fire (*Botrytis tulipae*) by building up a concentration of griseofulvin within the plant and then to cut sections and examine the tissues and hyphae microscopically to find out how the fungus was being controlled. Other experiments were carried out with onion epithelium to test the power of penetration of *Botrytis allii* in the presence of various concentrations of the antibiotic.

Method. Six Darwin tulips of the same (unknown) variety, which had been grown outside to the stage of a 2-inch (5 cm.) green spike, were transferred with a small ball of soil into 6-inch pots containing a vermiculite-sand mixture (equal volumes) and placed in a warm greenhouse. The bulbs were watered three times weekly, once with tap water and twice with a liquid feed prepared from Mullard Nutrient Pdr. M. 15 as supplied by Mullard Horticultural Eng. Co. Ltd., Egham, Surrey. The bulbs were grown on to the green-bud stage (approx. 5 weeks), during which period three of the bulbs were each watered seven times with 50 ml. of water containing 85 $\mu\text{g./ml.}$ of griseofulvin, previously dissolved in a small quantity of acetone. The

addition of the griseofulvin in acetone to the water was made immediately before pouring the solution on to the soil surface. The leaves were not wetted by the solution. The control plants were each watered with 50 ml. of tap water containing the same concentration of acetone as the griseofulvin watered plants (0.85 per cent.). Watering with the griseofulvin and control solution was carried out on days when normal watering and feeding were not being done.

At the green-bud stage all the plants were severed, by means of sterile razor-blade, from the bulb at a point about 50–60 mm. above its tip. A drop of water was added to the cut surface of all the plants remaining in the pots and these surfaces were inoculated with spores of *B. tulipae* obtained from an infected plant. The plants were then transferred to a 'Perspex' humidity chamber in the greenhouse for 48 hours.

The upper parts of the plants were used to assay the griseofulvin in them. This was done by cutting off portions of the stem of each plant and, after weighing them, grinding them up with ten times their own weight of distilled water. The liquid so obtained was filtered and assayed with *B. allii*, as described by Brian, Curtis, and Hemming (1946). This method was used in preference to the improved method described by Crowdy and Pramer (1955), because it was thought that grinding up in water might give a truer measure of the free griseofulvin in the plant tissues. There is no evidence that bound griseofulvin would have the same effect on the invading fungus. Assayed in this way the upper stems of the treated tulips showed a concentration of between 2 and 3 $\mu\text{g./g.}$ (live wt.). The control plants on assay showed no trace of griseofulvin.

After 48 hours in the damp chamber the plants were removed: judging by the discoloration of the stems of the control plants, the fungus had advanced 28–34 mm. down the stems. The treated plants showed a shallow brown ring extending 2–3 mm. down the side of the stem, and this proved to be the total advance made by the pathogen.

The pieces of stem were fixed in Karpechenko's solution and embedded in paraffin wax; longitudinal sections were then made and stained in cotton-blue lactophenol. Sections of the control plants showed that the spores had germinated and the hyphae had grown down into the stem, passing freely from cell to cell, Pl. IX, F. There was some evidence of hyphae growing down xylem vessels, but this seemed to be unusual. Some distance behind the advancing hyphae the mycelium consolidated into a compact mass, which moved down the stem, leaving behind it complete cellular disintegration.

Sections from the treated plants showed that after germination the hyphae had grown down into the stem, gaining access to the cut cells at the surface. The griseofulvin in these cells was probably diluted by the drop of water placed on the cut surface. In the main the hyphae of *B. tulipae* were halted at the line of the first or second end walls of cells they encountered, and few hyphae were found beyond this region, Pl. IX, J. Some degree of penetration had taken place from the pathogen's growing through the intercellular spaces,

and this probably accounted for the pieces of mycelium found below the general line at which the fungus was halted. The xylem vessels were not used on this occasion, presumably because the concentration of griseofulvin was higher in them than in the surrounding tissues.

Individual hyphae of *B. tulipae* on encountering the walls of cells were usually turned by them, whereupon the hyphae proceeded to grow and form a spiral round the inside of the cell, until it became more or less full, Pl. IX, L. In some instances the hyphal tip on meeting the cell membrane stopped growing and a swelling formed a short distance behind the tip, Pl. IX, K. This was often followed by branching farther back, some of the branches growing round inside the cell and filling it up. Both stunting and curling of hyphae were seen in these sections, suggesting that, although the concentration of griseofulvin within the stem may have varied, the overall effective concentration of the antibiotic must have been in the neighbourhood of the transition concentration between curling and stunting. In vitro for *B. tulipae*, this is in the region of 1 $\mu\text{g./ml.}$, which confirms the figure obtained in the assay of different portions of the stem.

Penetration experiments using onion epithelium. Experiments were conducted to test the powers of *B. allii* to penetrate onion epithelium in the presence of griseofulvin. Onion epithelium was used because it can be stripped off the scales in large sheets, which are uniformly one cell thick. It can be easily handled without tearing or breaking. Further, the inside (adaxial) epithelium can be used, and it has no stomata or other openings that might allow false penetration. It was found that the 'toughness' of the epithelium decreased somewhat on the scales nearer the centre of the bulb and therefore the epithelium from one scale was used for one series of experiments. To provide the maximum amount of material from one bulb scale, large Spanish type onions were used.

These experiments were carried out in two ways. The first was to strip off several pieces of epithelium, approx. 2 cm. wide and 6–8 cm. long, and lay them in Petri-dishes previously prepared with 2½ per cent. malt agar containing known concentrations of griseofulvin. The inside surface of the epithelium was placed on the agar and smoothed flat with a sterile wire loop. The surface of the onion tissue was inoculated with *B. allii* spores and incubated at 24° C. until required. The second method was to cut slices of onion scale 2.5 cm. wide complete with epithelium and to soak the scale in sterile saline containing the desired strength of griseofulvin. After soaking, the pieces of scale were washed in saline for 1 minute and dried with sterile filter paper; the concave surfaces were inoculated with *B. allii* spores. The pieces were placed in Petri-dishes with damp cotton wool and incubated as described above. Controls were prepared by soaking pieces of onion scale in saline containing no griseofulvin.

After incubation the epithelium was stripped off the surface of the agar and the onion scales were stained in cotton-blue lactophenol and examined microscopically. The agar surface and the surface of the scale from which the

epithelium had been removed were also stained. Any mycelium that had penetrated the epithelium and grown either into the agar or into the tissue of the onion scale was left behind on removal of the epithelium and could be seen under the microscope. This together with examination of the stained epithelium provided an accurate assessment of the penetration, as well as information on the behaviour of the germ-tubes when inside the epithelial cells in the presence of griseofulvin.

The method involving placing the epithelium on the agar worked satisfactorily. The cells were not plasmolysed and remained healthy for at least three days. The difficulty with the method involving the whole onion scale lay in the length of time the tissue had to be soaked in griseofulvin solution to get an even distribution of the antibiotic within the tissues, from 24 to 48 hours. The variation appeared to be due to the difference in the condition of the scales: consequently all pieces were soaked for 48 hours. In spite of this, complete uniformity was not always achieved. Generally, however, the results obtained by the two methods were closely similar.

The concentrations of griseofulvin tested in these experiments and the results obtained are shown in Table I.

TABLE I
Penetration of Onion Epithelium by B. allii in the Presence of Griseofulvin

Griseofulvin $\mu\text{g./ml.}$	12 hours	24 hours	48 hours	72 hours
30	—	—	—	—
15	—	—	—	—
10	—	—	±	+
5	—	—	+	++
3	—	±	++	++
1	±	+	++	++
0.3	+	++	++	++
0.0	++	++	++	++

— = no sign of penetration.

± = a few isolated cases of penetration.

+

++ = vigorous penetration.

These results show that under the above conditions a concentration of between 10 and 15 $\mu\text{g./ml.}$ of griseofulvin prevents the hyphae from penetrating the epithelium for three days. Lower concentrations cause delay for varying periods. After germination, facile penetration of the outer cell membrane occurs. Once inside the cell at griseofulvin levels of 3–5 $\mu\text{g./ml.}$ and above, development of the fungus is severely reduced. At about 1.0 $\mu\text{g./ml.}$ the intra-cellular hyphae are somewhat stunted but growth continues at a reduced rate. Concentrations below this cause curling, but penetration does not appear to be affected.

Discussion. Results both from *in vitro* and *in vivo* experiments have shown that the fungal hypha is profoundly modified when growing in physical

contact with griseofulvin. These modified hyphae are less able to penetrate plant cell membranes than those growing in the absence of griseofulvin.

Systemic concentrations of the antibiotic only sufficient to cause curling of *Botrytis* hyphae may not be capable of giving full protection to the plant against these pathogens. If, however, a concentration of griseofulvin can be built up inside the plant sufficient to cause stunting ($> 1 \mu\text{g./ml.}$), it would seem that protection of the plant against attack from *Botrytis* would be achieved. The presence of the antibiotic within the cells of the plant allows spore germination and possible penetration of the epidermal layers by a pathogen, but would, if the concentration were sufficient, restrict that pathogen to a few adjacent cells. Griseofulvin applied by spray would act on the fungus on germination, the stage at which it is most vulnerable to the antibiotic. Information on the amount of griseofulvin absorbed by a plant sprayed with it would be valuable.

SUMMARY

1. *In vitro* and *in vivo* experiments have been carried out to find out how griseofulvin affects the growing hyphae of *Botrytis* species.

2. *In vitro* experiments to study the reaction of growing *Botrytis* hyphae on entering a griseofulvin environment have shown that the antibiotic only acts on a small region of the hypha around the tip and that it is not translocated within the mycelium. Some modification of the protoplasmic content of hyphae takes place together with loss of rigidity of hyphal membranes.

3. *In vivo* experiments, involving control of the spread of *B. tulipae* in tulip plants by griseofulvin taken into the plant via the roots, showed on sectioning the tissues that the fungus is unable to pass from cell to cell through the cell membranes in the presence of the antibiotic and so is unable to spread through the plant.

4. Experiments to test the ability of *B. allii* to penetrate onion epithelium in the presence of griseofulvin showed that penetration is delayed for 3 days by 10–15 $\mu\text{g./ml.}$ of the antibiotic. Hyphae within the epithelial cells are profoundly modified in form.

5. All the hyphal modifications observed *in vitro*, such as curling, stunting, and bursting of hyphae, have been observed *in vivo*.

ACKNOWLEDGEMENTS

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EXPLANATION OF PLATES

Illustrating R. S. C. Aytoun's article on 'The Effects of Griseofulvin on Certain Phytopathogenic Fungi'.

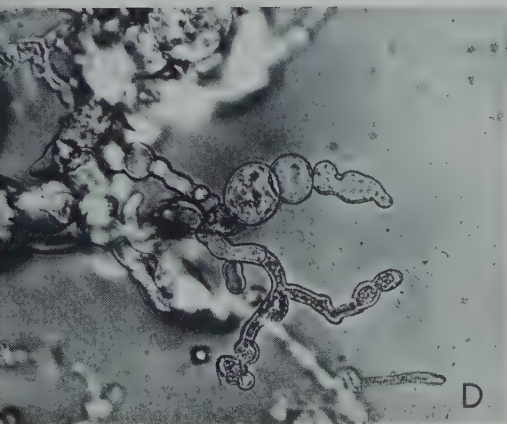
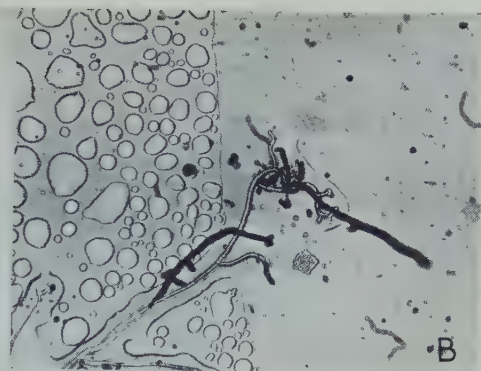
(Note: The magnifications are approximate.)

PLATE VIII 'in vitro'

- A. Hypha of *Botrytis tulipae* entering a malt agar hanging drop containing no griseofulvin. Note rate of branching. ($\times 100$.)
- B. Hypha of *B. tulipae* entering a hanging drop containing $1.6 \mu\text{g./ml.}$ of griseofulvin. Showing early stimulation to branch with loss of direction of growth. Dark aerial hyphae not in contact with the medium appear normal. ($\times 100$.)
- C. Hypha of *B. tulipae* entering a hanging drop containing $12 \mu\text{g./ml.}$ of griseofulvin. Shows immediate stimulation to profuse branching. Hyphae have granular contents and are of uneven thickness. ($\times 500$.)
- D. Hyphae of *B. cinerea* after growing for 48 hours in an environment containing $11 \mu\text{g./ml.}$ of griseofulvin. Shows breakdown in rigidity of hyphal walls causing random swelling of the mycelium, with granular contents. ($\times 500$.)
- E. Showing the typical bursting of a hypha near the tip, frequently seen when hyphae are in contact with concentrations of griseofulvin of about $10 \mu\text{g./ml.}$ or more. ($\times 500$.)

PLATE IX 'in vivo'

- F. Hyphae of *B. tulipae* advancing down inside the stem of an untreated tulip. Hyphae are straight and pass easily from cell to cell. (Compare with G.) ($\times 500$.)
- G. Section of tulip stem treated with griseofulvin and containing about $2.0 \mu\text{g./gm.}$ live weight. Advancing hyphae of *B. tulipae* show obvious curling and twisting. ($\times 500$.)
- H. Section of untreated tulip stem at the cut surface inoculated with *B. tulipae*. Hyphae are seen throughout the section and are able to pass readily from cell to cell. (Compare with J.) ($\times 100$.)
- J. Section of treated tulip stem at the cut surface where inoculation took place. Advance of *B. tulipae* is stopped by first end walls of cells, which it cannot penetrate. ($\times 100$.)
- K. Showing hyphae of *B. tulipae* stopped by the end wall of a cell in the stem of a treated tulip. Note swellings just behind the tips of some hyphae. ($\times 500$.)
- L. Cell in stem of tulip treated with griseofulvin that has contained the pathogen. Hyphae of *B. tulipae* have almost filled the interior of the cell and are unable to break out. ($\times 500$.)





Studies on the Nutrition of *Coprinus lagopus* Fr., especially as affecting Fruiting

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With four Figures in the Text

ABSTRACT

Growth and fruiting of *Coprinus lagopus* in pure culture have been studied on a medium containing glucose, dl- α -alanine, mineral salts and thiamin, and the effects of changes in concentration of certain components of this medium and of various substitutions investigated. It is demonstrated that fruiting led to withdrawal of materials from the whole of the colony. No evidence was obtained of the existence of a minimal quantity of substrate for fruiting to take place. The distribution of mature sporophores on a colony was affected by an internal mechanism. A theory as to its possible nature is suggested.

I. INTRODUCTION

INVESTIGATIONS on the nutrition of Hymenomycetes and its effect on fruiting are still few in number compared with those for other major groups of fungi. Nutritional requirements of *Coprinus lagopus* have been studied to some extent by earlier workers, but in certain respects there are apparent discrepancies in the results obtained. Voderberg (1948) found that growth and fruiting of this organism in partially chemically defined media were dependent on the presence of cellulose in the form of finely ground straw or blotting-paper. The presence of powdered straw appeared to permit some use of simpler carbohydrates which the fungus was otherwise unable to use. Voderberg also reported that nitrate-, ammonium-, and organic-nitrogen could be used, although growth and fruiting were best with organic-nitrogen sources. On the other hand, Leonian and Lilly (1938) successfully cultured the mycelium of *C. lagopus* on an agar medium containing glucose as the carbon source and nitrogen as a mixture of amino-acids. These workers reported that neither ammonium- nor nitrate-nitrogen was used, and that thiamin was the only vitamin requirement. No fruiting was reported on any of the media tested.

Voderberg (1949) concluded that the initiation of fruiting in *C. lagopus* was independent of the quantity of available food substances since cultures on different quantities of manure gave rise to sporophore primordia simultaneously. On the other hand, a certain amount of mycelium, corresponding to that formed on at least 1.5 g. of manure, was indispensable. This result is compatible with Buller's view (1931, p. 152 et seq.) that fruiting could take place in the larger fungi only when there was sufficient vegetative mycelium to support a fruit-body of a certain minimal size.

In a number of fungi exhaustion of the substrate has been found to be conducive to the initiation of fruiting. Wakefield (1909) found that this was so for *Schizophyllum commune* and *Stereum purpureum* grown in pure culture. Other Hymenomycetes, too, fruit earlier with low initial concentrations of sugar in the medium than with high (Plunkett, 1953; Bille-Hansen, 1953).

2. MATERIAL AND METHODS

(a) *Origin and identity of the isolate.* The organism used throughout this work was established in pure culture from spores shed by a mature fructification which developed on horse dung incubated in the laboratory.

There is a good deal of confusion concerning the nomenclature of *Coprinus lagopus* and allied forms. The difficulty in naming these organisms stems from their morphological similarity and also from conflicting statements in the taxonomic literature on their coprophilous habit. In published experimental work on the Hymenomycetes the name *C. lagopus* has been applied to a coprophilous fungus by a number of workers (Brefeld, 1877; Buller, 1924; Borriass, 1934*a, b*; Voderberg, 1948, 1949, 1950), and it appears from illustrations in several of these works that these organisms were *C. lagopus* as understood by taxonomists such as Lange (1915, 1935), Konrad and Maublanc (1924–37), and Rea (1922), although only Rea records this species as occurring on dung.

Sporophores produced by cultures on sterilized horse dung agreed well with the emended description of *C. lagopus* given by Buller (1924). The isolate further resembled those of Buller in occasionally producing dwarf fruit-bodies, sometimes little more than 1 cm. high and slender in proportion (Buller, 1922, 1924). It was therefore concluded that the organism used lay within Buller's concept of *C. lagopus*, and his example in the use of the name was followed.

(b) *Cultural techniques.* Preliminary experiments indicated that growth of the organism was reduced by an initial pH less than 6.5 and inhibited below pH 4.5. The following medium had a favourable reaction and supported dense vegetative growth and good fruiting. It was adopted as the medium on which stock cultures of the isolate were maintained throughout this work.

0.3 per cent. Yeast Extract Agar

Glucose	.	.	.	.	1%
Yeast extract ¹	.	.	.	.	0.3%
K ₂ HPO ₄	.	.	.	.	0.2%
MgSO ₄ ·7H ₂ O	.	.	.	.	0.02%
Agar	.	.	.	.	2%

An analysis of the yeast extract, kindly supplied by the manufacturer,¹ showed that it contained considerable quantities of amino-acids. A number of trials revealed that dl- α -alanine could function as the nitrogen source in place of the yeast extract if an external source of thiamin was available. Accordingly the above medium was modified for use in experimental work, the yeast

¹ 'Yeastrel', manufactured by the Brewers' Foods Supply Co. Ltd., Edinburgh.

extract being replaced by 0.1 per cent. dl- α -alanine and thiamin hydrochloride at a concentration of 500 μ g. per 1,000 ml. of medium. This medium will be subsequently referred to in the text as 'Agar Medium A', or if the agar is omitted, 'Liquid Medium A'. In experiments in which the nutrients were varied, the basal medium will be designated, for example, as 'Agar Medium A less Glucose'. Wherever possible, chemicals of analytical reagent quality were used, although no differences were detected between growth in Liquid Medium A prepared from ordinary laboratory chemicals and in that prepared from analytical reagents.

The culture vessels used were 10 oz. medicine bottles (approx. $13 \times 6 \times 3.5$ cm.) lying flat on their sides, with necks plugged with cotton wool. Each contained 20 ml. of medium, liquid or agar.

Since the medium was slightly alkaline, the caramelization of the glucose which would have occurred on autoclaving, and might have influenced the growth of the fungus, was avoided or minimized throughout this work by sterilizing media either by steaming for half an hour on three successive days or by autoclaving the glucose and phosphate separately and combining them later aseptically.

C. lagopus can use the thiazole and pyrimidine moieties of thiamin which are formed when thiamin undergoes alkaline hydrolysis (Leonian and Lilly, 1938); thiamin could therefore be added to the rest of the medium before sterilizing or afterwards. Usually it was added prior to sterilization.

Mycelial inocula were always used. Plugs of agar, 1 mm. in diameter, bearing vegetative mycelium, were removed at a distance of approximately 3 mm. within the margin of a plate culture on 0.3 per cent. Yeast Extract Agar, and one was placed in each culture vessel. In agar cultures it was placed centrally upon the medium unless otherwise stated in the text. The age of the stock culture at the time of removing the 1 mm. plugs was usually between one and two weeks.

Cultures were incubated at 25° C. with continuous illumination at about 8 foot candles provided by an incandescent filament bulb.

(c) *Methods of assessing growth and fruiting.* Radial growth of fungal colonies was determined from measurements of two diameters along the diagonals of the rectangular medicine bottles. Additional data on growth and fruiting were obtained by determining the dry weight of mycelium and of fruit-bodies.

With cultures on liquid media this presented little difficulty. After dissecting away all fruit-bodies, the mycelia were carefully washed in a Gooch crucible, dried at 70° C. for 2 days and then weighed to 0.1 mg. However, with colonies grown on agar media it was first necessary to free the mycelium from the substrate. A modification of the method of Hall (1933) was adopted. An excess of hot water was added to the culture which was then heated in a steamer at 100° C. for 20 to 30 minutes. The freed disc of matted hyphae was then carefully and thoroughly washed in several changes of boiling water, dried and weighed. It was appreciated that this treatment was liable to result

in a loss of weight by the escape of soluble materials through cell membranes made permeable by killing, but this loss, and therefore the weight obtained, was assumed to be proportional to the true dry weight of the mycelium. The magnitude of the loss in weight caused by this treatment was investigated by comparing the dry weights of 23 desiccator-dried, 13 days old mycelia from liquid cultures before and after heating in tubes of water in a steamer at 100° C. for 30 minutes. The mean loss after this treatment was 21.3 ± 0.88 per cent. of the initial dry weight. This should be borne in mind in considering the significance of total weights given in following experiments, where true dry weights of the sporophores are summed with dry weights of mycelia obtained in the way described above.

Yields of sporophores were, as indicated above, recorded gravimetrically. This method was found to be the most satisfactory, since replicates often differed in number and size of sporophores, yet in terms of total dry weight of sporophore tissue agreed closely. In certain cases further data qualifying fruiting are presented. No difficulty was experienced in separating fruit-bodies from the vegetative mycelium, even in their primordial stage.

Differences significant at the 5 and 1 per cent. levels are given for most data; for certain others, the standard error of the mean is given as a measure of their dispersion. Since dry weights of mycelium and sporophore tissue are complementary within the total weight, generally only two of these three sets of dry weights are treated statistically.

Other techniques are described in appropriate places in the text.

3. EXPERIMENTAL RESULTS

(a) *Sources of carbon.* To determine which organic compounds could be utilized as sources of carbon by *C. lagopus*, a number (Table I) were separately incorporated in a basal medium of Agar Medium A less Glucose. Each was added at a concentration of 1 per cent. W/V. Since a low level of soluble carbohydrate might have been necessary to initiate growth on media containing insoluble polysaccharides, starch and cellulose were each tested with and without 0.1 per cent. glucose. The cellulose used in this experiment was mechanically macerated and well-washed filter paper.

Since a number of treatments had by the 14th day produced large crops of sporophores which would have deliquesced had they been left longer, fruit-bodies from these were harvested at that date and the yields of mycelium determined after melting the agar. The more slowly developing treatments were left for 20 days before harvesting. The results are presented in Table Ia. A treatment with sucrose as the carbon source was spoiled by contamination. The utilization of this carbohydrate was therefore investigated separately in a similar fashion, and the results given in Table Ib. When cellulose was used it was virtually impossible to free the hyphae from the fibres; the dry weights determined in such cases are therefore omitted from the data.

Assessed visually, growth on cellulose media was good both with and with-

out additional glucose. The tabulated results show that the sources of carbon tested fall into three main categories, namely:

- (i) those which were used readily and gave good vegetative growth and fruiting; all are carbohydrates of various types.
- (ii) those which were used to a very limited extent or not at all, and did not support fruiting even though incubated for a longer period than the sources in category (i).
- (iii) those which actually inhibited growth; the presence of galactose and lactose in this group is particularly interesting.

TABLE I

The Effect of Source of Carbon on Growth and Fruiting of Coprinus lagopus

(a) Three replicates

Source of carbon	Period of incubation (days)	Mean dry weight (mg.)			Mean days to fruit†
		Mycelium	Sporophores	Total*	
Glucose	14	46.6	25.6 ± 2.3	72.2	9.7 ± 0.3
Maltose	14	33.6	20.9 ± 1.2	54.5	7.3 ± 0.9
Fructose	14	35.8	18.6 ± 3.9	54.4	9.3 ± 0.3
Starch + 0.1% glucose	14	36.7	15.9 ± 1.9	52.5	7.7 ± 0.3
Starch	14	26.7	10.2 ± 0.7	36.9	8.0 ± 0.6
dl-α-alanine	20	22.7	0	22.7	—
0.1% glucose	20	20.9	0†	20.9	20.0 ± 0
Glycerol	20	18.8	0	18.8	—
Na-K-tartrate	20	14.2	0	14.2	—
NO ADDITION	20	10.8	0	10.8	—
Galactose	20	1.1	0	1.1	—
Lactose	20	1.1	0	1.1	—
Na-oleate	20	0	0	0	—
Cellulose	20	—	12.0 ± 2.8	—	12.0 ± 0
Cellulose + 0.1% glucose	20	—	16.5 ± 2.6	—	12.0 ± 0

* Significant differences between means of total fungus weight ($P = 0.05$, 4.7 mg. $P = 0.01$, 6.4 mg.

† In this and other tables, 'days to fruit' means until the first sporophore primordia appear. A dash, in this column, means none appeared by end of experiment.

‡ Primordia present, but of negligible weight.

(b) Four replicates

Source of carbon	Period of incubation (days)	Mean dry weight (mg.)		
		Mycelium	Sporophores	Total*
NO ADDITION	28	16.8	0.3	17.1
Sucrose	28	18.6	0	18.6

* Difference between means of total fungus weight not significant ($P = > 0.2$).

The ready utilization of starch and cellulose was confirmed by repeating part of the above experiment. An iodine test on starch-containing media on which *C. lagopus* had grown revealed the absence of starch in the immediate vicinity of the colony.

In the hope that some light might be shed on the mechanism underlying

the inhibitory properties of galactose and lactose, the effects of these and of two related sugars were studied further. The additional sugars were xylose (a pentose sugar in which the configuration of part of the molecule is similar to that in galactose) and raffinose (a trisaccharide containing a galactose residue). These four sugars were Seitz-filtered and separately added to triple-steam-sterilized Agar Medium A less Glucose at a final concentration of 1 per cent. In a second series, glucose at 0.5 per cent. was added to each to see if any inhibition that these sugars might produce could be offset by the presence of a readily utilized carbohydrate.

TABLE II

The Effect of Galactose and Allied Sugars on Growth and Fruiting of Coprinus lagopus

Sugar	Incubation period 15 days. Four replicates							
	Without glucose				With 0.5% glucose			
	Mean dry weight (mg.)			Days to fruit	Mean dry weight (mg.)			Days to fruit
	Myc.	Sporo.	Total*		Myc.	Sporo.	Total*	
Galactose	1.7	0	1.7	—	65.8	0 (+)	65.8	5.5 ±0.3
Lactose	1.6	0	1.6	—	27.5	0	27.5	—
Raffinose	11.2	0	11.2	—	24.5	20.9 ±1.8	45.4	6.5 ±0.3
Xylose	20.5	23.7 ±0.7	44.2	7.0 ±0	93.4	0.6 ±0.2	94.0	7.3 ±0.3
Nil	10.2	0	10.2	—	25.1	19.0 ±1.3	44.1	6.8 ±0.3

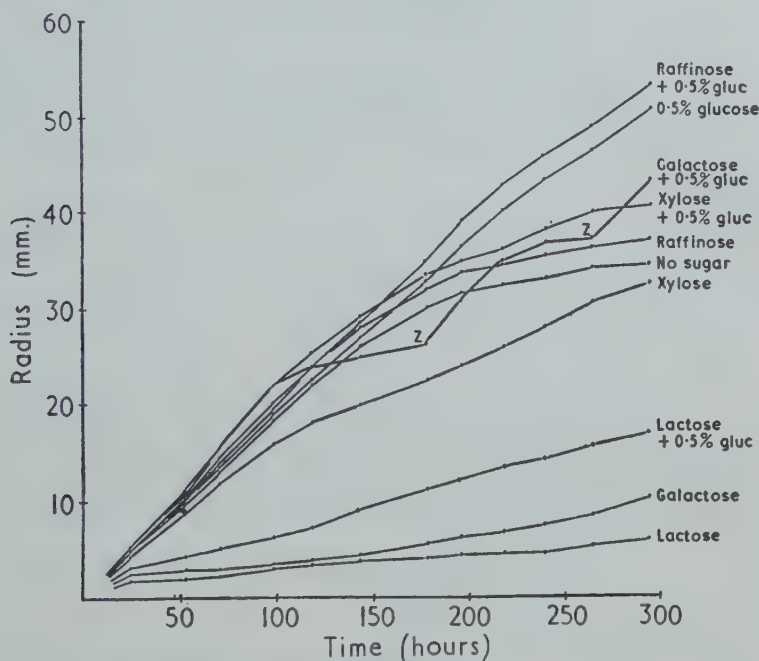
* Significant difference between means of total fungus weight ($P = 0.05$, 3.6 mg.
 $P = 0.01$, 4.9 mg.

(+) Indicates fruiting commenced, but was negligible.

The results in Table II confirm the effects of galactose and lactose noted above, but show that neither xylose nor raffinose was inhibitory. Xylose was in fact readily utilized while raffinose neither stimulated nor inhibited growth. However, in the presence of glucose, galactose increased growth and colonies showed a pronounced zonation which was reflected in the changing rate of radial growth (see Text-fig. 1). Lactose retained some inhibitory properties in the presence of glucose, for the growth, although dense, was restricted in its radial extent. With glucose, the fungus was again unaffected by raffinose, while xylose supported a large yield of vegetative mycelium. It is interesting to note the opposite response of vegetative and reproductive growth when 0.5 per cent. glucose was added to xylose; vegetative growth increased more than fourfold while fruiting was almost suppressed. The added glucose did not evoke the same response in all cases, for in the 'Nil' treatments it increased both vegetative and reproductive growth. These results are discussed later.

The zonation on galactose-glucose medium was produced by a progressive intensification of the density of mycelial growth, culminating in the formation of sporophore primordia just within the margin of the colony, and

followed by an abrupt change to a very thin, rapidly spreading vegetative growth. Since this change followed so soon after primordia were formed, it was possible that initiation of primordia might have been a direct response to conditions in the medium, and the subsequent decrease in the density of vegetative growth a consequence of this. A similar, though less marked, reduction of the density of mycelial growth followed a day or two after sporophore initiation in fruiting cultures on other media in this and other experiments.



TEXT-FIG. 1. The effects of different carbon sources on radial growth of colonies of *Coprinus lagopus*. Z indicates each transition from dense growth to sparse mycelium in colonies cultured on medium with galactose and 0.5% glucose.

The following experiment was conducted to test the possibility. Advantage was taken of preliminary results which indicated that fruiting could be retarded by withholding light. Twelve cultures were set up on galactose-glucose medium of the same composition as before. Four were kept in continuous light and the remainder in continuous darkness. Of the latter, four were examined on the 12th day and four on the 16th. It was found that in all cases the vegetative mycelium was prominently zoned, but only in continuous light were there any sporophore primordia. Although all twelve cultures were kept for 2 months, no primordia developed into more than minute, stunted sporophores.

(b) *The effects of different glucose concentrations.* Glucose was added to Agar Medium-A less Glucose to give initial concentrations of 0.6 per cent. The media were adjusted to pH 8.0 with sodium hydroxide solution and

sterilized by steaming on three successive days. Radial growth was measured up to the 17th day and the yields of mycelium and sporophore tissue then determined.

The results (Table III) demonstrate that fruiting was more specific in its requirements than was vegetative growth, and that the optimal glucose concentrations for the two processes were different. The sporophore crops produced at 0.75 and 1 per cent. concentrations included mature specimens. Sporophores at lower concentrations were immature and smaller. Above 1 per cent. glucose, none had progressed beyond the primordial stage. At 2 per cent. glucose, all replicates bore primordia; at 3 per cent., only one replicate began to fruit, and had a negligible quantity of sporophore tissue; at 4 per cent. and above, no primordia were formed. In Table III, the non-fruiting replicates are included when calculating the mean yields of sporophore tissue.

The rate of radial growth (Text-fig. 2) fell as the initial concentration of glucose was increased. The growth rate with 0 per cent. glucose, which was at first the greatest, diminished rapidly at about the 10th day.

Although high initial concentrations of glucose delayed the appearance of the first primordia (Table III) it is unlikely that the low yields of sporophore tissue obtained in such cases were the result of premature harvesting. It is doubtful whether the primordia produced at 2 and 3 per cent. glucose would have matured, for their rate of development, so far as it was observed, was very slow. Thus the diminution of yield at supraoptimal concentrations of glucose can only in small part be attributed to the delay in the appearance of the primordia.

The above experiment was repeated with Liquid Medium A less Glucose, when similar relationships between vegetative growth, fruiting and glucose concentration were obtained.

(c) *Sources of nitrogen.* Various nitrogenous materials were added to Agar Medium A less Alanine; the final concentration of nitrogen in each treatment was the same and equivalent to M/30 sodium nitrate. Since the utilization of nitrogen commonly leads to changes of pH of the medium, and preliminary work had indicated that acid conditions adversely affected the growth of *C. lagopus*, each nitrogen source was tested alone and with 1 per cent. calcium carbonate. Glucose was autoclaved separately and then added aseptically to the rest of the medium. Before determining their dry weights, all mycelia were quickly washed in dilute (approx. 0.5 N.) hydrochloric acid, to remove such insoluble calcium salts as may have been adherent. The results are presented in Table IV.

Adopting the total yield of fungus as a measure of the efficiency with which the nitrogen source was used, it is evident that *C. lagopus* was able to make little or no use of nitrate-nitrogen. Good growth was made upon media containing ammonium salts without calcium carbonate, although there was no fruiting. Yields with the three ammonium salts were greatly increased ($P = < 0.01$) by the presence of calcium carbonate, and differed highly significantly

TABLE III

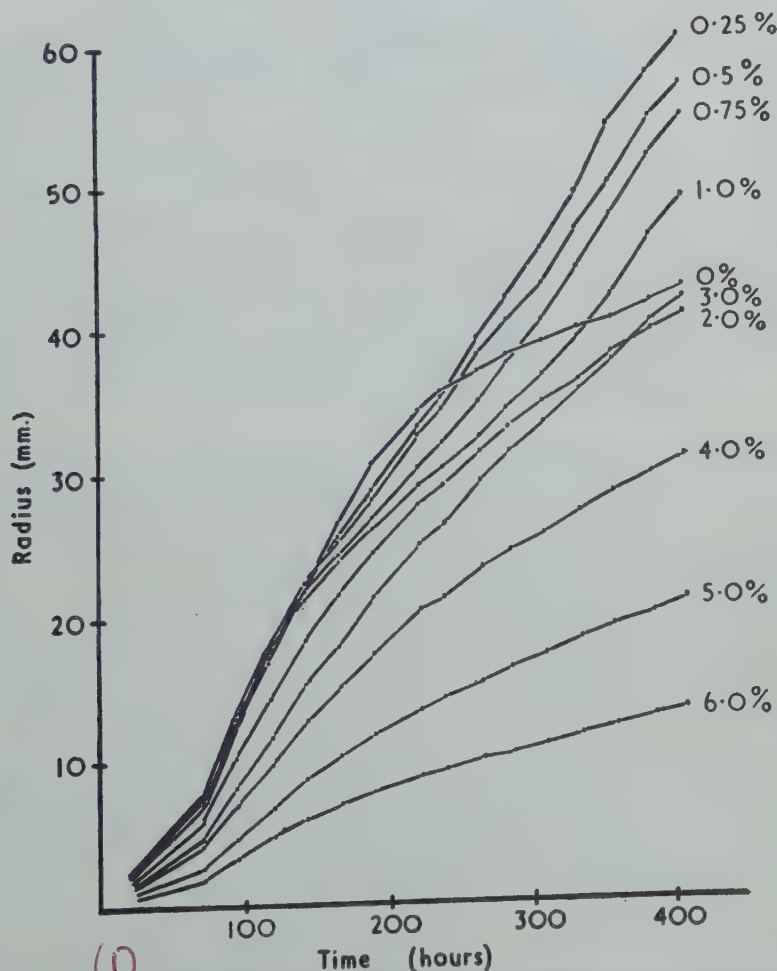
*The Effects of Different Initial Concentrations of Glucose on Growth and Fruiting of Coprinus lagopus*Incubation period 17 days. Four replicates
Initial Concentration of Glucose (%)

D.W. (mg.)	0	0.25	0.5	0.75	1.0	2.0	3.0	4.0	5.0	6.0
Myc.*	5.5	14.7	19.3	21.7	34.2	107.6	111.7	75.2	34.4	12.7
Sporo.*	0	9.3	15.8	22.0	23.4	0.2	0	0	0	0
Total†	5.5	24.0	35.1	43.7	57.6	107.8	111.7	75.2	34.4	12.7
Days to fruit	15->17†	9.3	9.3	9.0	10.3	13.8	14->17	>17	>17	>17
		±0.3	±0.9	±0.4	±0.3	±0.5				

* Significant difference between means of sporophore dry weight { $P = 0.05$, 4.3 mg.
 $P = 0.01$, 5.9 mg.

† Significant difference between means of total dry weight { $P = 0.05$, 12.9 mg.
 $P = 0.01$, 17.4 mg.

‡ One or more replicates produced primordia on the 15th day, but some were still sterile at the end of the experiment on the 17th day.



TEXT-FIG. 2. The effects of different initial glucose concentrations on radial growth of colonies of *Coprinus lagopus*.

($P = <0.01$), while fruiting was then prolific with two of them. Alanine was readily used, but in this experiment fruiting was negligible; this was unusual, for normally it permitted fruiting to commence on or about the 10th day. Peptone also supported good growth; here however growth was reduced ($P = <0.01$) in the presence of calcium carbonate. Although the final dry weights of cultures from media containing glycine or asparagine were small, growth in both cases was very dense but restricted in diameter (Table IV). This contrasts with the control treatment in which, although the yield was much the same, the diameter was approximately double, and the mycelium very sparse.

TABLE IV

The Effect of Source of Nitrogen on Growth and Fruiting of Coprinus lagopus

Source of nitrogen	Incubation period 12 days. Four replicates									
	Without 1% CaCO ₃			Days to fruit	Mean diam. 9th day (mm.)	With 1% CaCO ₃			Mean diam. 9th day (mm.)	
	Mean D.W. (mg.)					Mean D.W. (mg.)				
	Myc.	Sporo.	Total*			Myc.	Sporo.	Total*		
NO ADDITION	8.4	0	8.4	—	72	13.9	0	13.9	—	73
Sodium nitrate	10.8	0	10.8	—	62	11.8	0	11.8	—	66
Ammonium chloride	23.4	0	23.4	—	71	35.2	19.3	54.5	9.5	79
Ammonium tartrate	21.5	0	21.5	—	65	35.5	±8.4	35.5	—	63
Ammonium nitrate	25.2	0	25.2	—	75	20.1	50.2	70.2	9.0	84
Glycine	10.4	0	10.4	—	27	9.2	±4.4	9.2	±0.6	29
Alanine	33.9	0	33.9	10—>12	66	32.1	0	32.1	12—>12	62
Asparagine	16.7	0	16.7	—	39	12.6	0	12.6	—	39
Peptone	36.2	24.8	61.2	9.5	69	14.6	0	14.6	—	35
		±1.8		±0.5						

* Significant difference between mean total dry weights $\left\{ \begin{array}{l} P = 0.05, 8.3 \text{ mg.} \\ P = 0.01, 11.0 \text{ mg.} \end{array} \right.$

The experiment was repeated, except for the treatments with glycine and peptone, and the above results confirmed. Urea was tested separately, both with and without the addition of calcium carbonate, and was found to support in both cases a moderately good growth of mycelium, but sporophores did not appear during the 12 days over which observations were made.

Since the adverse effect of calcium carbonate on the utilization of peptone was unexpected, it was further investigated. Peptone media, with and without calcium carbonate, were prepared as above and sterilized by autoclaving, while in a similar series of treatments the peptone was autoclaved separately and added aseptically afterwards. The results are given in Table V.

The interaction between presence of calcium carbonate and the method of sterilizing was not statistically significant. However, the aggregate means confirmed that calcium carbonate reduced the total yield of fungus ($P = <0.01$) and fruiting ($P = <0.01$) and showed that the method of sterilizing had no significant effects. This suggests that the adverse action of calcium carbonate was not caused by its reaction with peptone during autoclaving.

In some of the experiments reported above and in others, cultures on different media which fruited well differed not only in yield of sporophore tissue

but also in the number and size of the mature fructifications. This was particularly true when fruiting on Agar Medium A was compared with that on media whose compositions differed only in that the alanine was replaced by ammonium chloride or nitrate plus 1 per cent. CaCO_3 . In a particular instance,

TABLE V

The Effect of Calcium Carbonate and Mode of Sterilizing on Utilization of Peptone by Coprinus lagopus

Incubation period 10 days. Four replicates

		Mean dry weight (mg.)	
		Without CaCO_3	With CaCO_3
Peptone autoclaved separately	{ Mycelium	30.7	32.3
	{ Sporophores	21.3	0
	{ Total	52.0	32.3
Peptone not autoclaved separately	{ Mycelium	33.1	36.0
	{ Sporophores	28.2	3.6
	{ Total	61.3	39.6
Aggregate means.	{ Sporophores*	24.8	1.8
	{ Total†	56.6	36.0

* Significant difference between mean aggregate sporophore yields, $P = 0.01$, 13.4 mg.

† Significant difference between mean aggregate total yields, $P = 0.01$, 14.4 mg.

four cultures on Agar Medium A were compared with four on 0.133 per cent. (M/60) NH_4NO_3 + 1 per cent. CaCO_3 medium after incubation for 17 days. Only large fruit-bodies and very small primordia were present. It was estimated that the dry weights of the latter would be extremely small and that

TABLE VI

The Effect of the Composition of the Medium on the Number and Size of Sporophores Maturing in Cultures of Coprinus lagopus

Incubation period 17 days. Four replicates

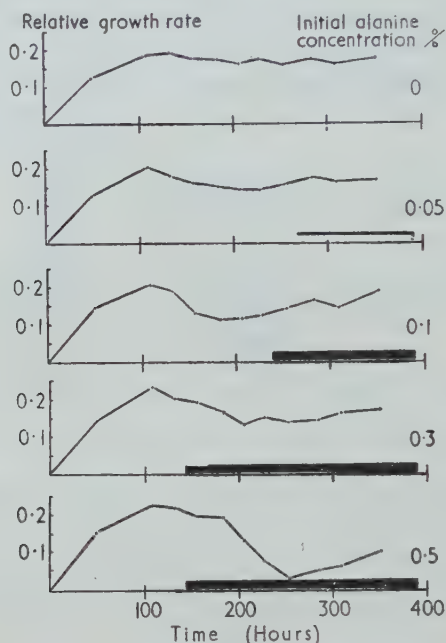
	Mean dry weight of sporophore tissue per culture (mg.)	Mean number of mature sporophores per culture	Mean dry weight of individual sporophore (mg.)
Alanine medium . . .	31.9 ± 4.3	5.3 ± 0.5	6.2 ± 1.0
$\text{NH}_4\text{NO}_3 + \text{CaCO}_3$ medium . . .	45.4 ± 3.4	3.0 ± 0.6	19.6 ± 2.8
Significance of differences between treatments: $P = < 0.05$			$P = < 0.01$.

they could be ignored in calculating the mean dry weights of the individual mature or nearly mature sporophores from the total yields of sporophore tissue in each treatment, as shown in Table VI. It is evident that the sporophores on the ammonium nitrate medium were on average approximately half as numerous but three times the dry weight of those on the alanine medium.

(d) *The effects of different alanine concentrations.* The initial concentration of dl- α -alanine was varied within the limits 0 and 0.5 per cent. in Agar Medium A less Alanine. To compensate for slight differences in pH resulting from the use of different quantities of alanine the reactions of all media were

adjusted to pH 8.0. Treatments were in quadruplicate. The results are presented in Text-figs. 3 and 4.

Text-fig. 3 shows that as the cultures aged the radial growth rate changed in a similar fashion at all levels of alanine. It rose rapidly to a maximum, then fell gradually and ultimately increased once again. Although the growth rate

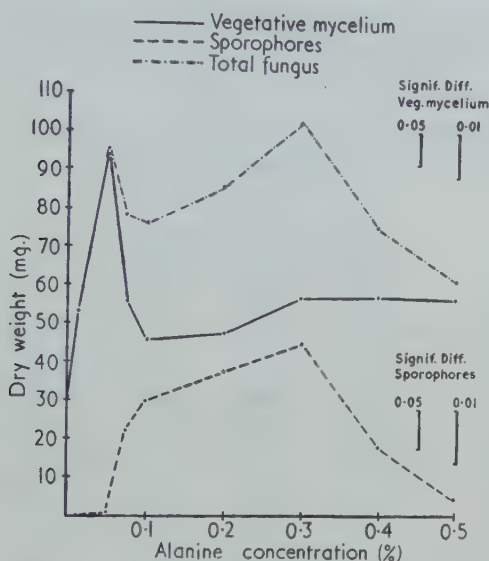


TEXT-FIG. 3. The effects of different initial concentrations of alanine on rate of radial growth and on the onset of fruiting of *Coprinus lagopus*. The horizontal lines indicate the period of fruiting from the first appearance of sporophore primordia in each of the four replicates of each treatment. Growth rate is expressed in millimetres per hour. (The figure does not include all the concentrations mentioned in the text.)

was initially slightly greater with the higher concentrations of alanine, it later diminished to a very low level. The onset of fruiting was accelerated by high levels of alanine, but bore no obvious relationship to the fluctuations of the growth rates. The density of mycelial growth in the early stages was increased perceptibly with each increment of alanine, at least over the range 0.05 per cent. and to a more limited extent later. A fringe in which aerial mycelium was sparse was usually clearly displayed around fruiting cultures about 2 days after the first primordia appeared. Later the mycelium grew with its original vigour.

The relations between dry weight of mycelium and alanine concentration appeared complex. Increases in the initial concentration of alanine up to 0.05 per cent. significantly increased the yield of mycelium, but immediately

above this level there was an abrupt and highly significant fall. With further increases in alanine concentration, the dry weight of mycelium increased slightly to reach a more or less uniform level at 0.3–0.5 per cent. alanine. The response of fruiting was simpler. The yield rose from a low level at 0.05 per cent. alanine to a maximum at 0.3 per cent., and was much reduced at higher concentrations. Some of the differences between yields of sporophores in the different treatments are attributable to the fact that all cultures were harvested



TEXT-FIG. 4. The effects of different initial alanine concentrations in agar medium upon mean dry weight yields of vegetative mycelium, sporophores and total fungus by *Coprinus lagopus* after incubation for 16 days.

(3)

simultaneously whereas fruiting began at different times (Text-fig. 3). Thus the media with 0 and 0.01 per cent. initial alanine had failed to fruit within the incubation period. Each replicate of the series 0.05, 0.075, 0.1, and 0.2 per cent. alanine showed respectively primordia, sporophores approaching maturity, and in the last two, mature sporophores. Using the blackish coloration of the pilei as an indication of fertility (Borriss, 1934a) the sporophores at or near maturity in these treatments were all fertile. Therefore, within the range of initial alanine concentrations 0 to 0.2 per cent., the variation in sporophore yield appeared to be largely due to differences in the times at which fruiting began. At 0.3 per cent. alanine, a similar explanation applies in part, but the sporophores failed to mature properly. A few almost attained mature form, but, judging by the cream colour of the pilei, they were not fertile, and the bulk of the yield of sporophore tissue was made up of a large number of small, immature sporophores. At 0.4 and 0.5 per cent. alanine, the yields of fruit-bodies were certainly not the result of delayed fruiting. In both

cases there was a very large number of primordia, but only at 0.4 per cent. alanine did any develop further and even these showed premature autolysis. Thus although fruiting commenced earliest in those treatments with high alanine, the development of the individual sporophores was retarded or even arrested.

An interesting relationship between vegetative and reproductive growth emerges from the data on dry-weight yields (Text-fig. 4), namely that the anomalous low yield of mycelium occurred at 0.075 per cent. alanine which was also the lowest concentration at which fruiting was of an appreciable extent.

TABLE VII

The Distribution of Fungous Tissue during Sporophore Formation by Cultures of Coprinus lagopus

Age of sample (days)	Four replicates Mean dry weight (mg.)			Stage of sporophore development
	Myc.	Sporo.	Total	
8.9 (9th)	26.0	0.2	26.2	Initiation of primordia
9.7 (10th)	30.1	0	30.1	
10.9 (11th)	31.6	3.9	35.5	Enlargement
11.7 (12th)	37.2	4.5	41.7	
12.7 (13th)	22.0	19.3	41.3	Maturation
13.7 (14th)	30.9	17.5	48.4	
15.0 (15th)	30.2	29.9	60.1	Deliquescence
16.1 (16th)	39.3	13.7	53.0	
16.7 (17th)	43.0	19.8	62.8	— } Second crop beginning

Significant differences

(mg.)				
between means	$P = 0.05,$	7.2	5.7	7.3
	$P = 0.01,$	9.7	7.7	9.9

A repetition of this experiment with Liquid Medium A less Alanine gave data which agreed essentially with the above, and again the peculiar drop in yield of mycelium was associated with the lowest alanine concentration at which fruiting was appreciable.

This association of low yield of vegetative mycelium with rapid sporophore development was interpreted as indicating that materials passed from the vegetative mycelium into the developing fruit-bodies. To test this interpretation, the growth of vegetative and sporophore tissues was followed gravimetrically by means of a random sampling technique. Thirty-six cultures were established on Agar Medium A from inocula placed on the medium half-way along one side of each medical flat, so permitting a longer period of uninterrupted growth than was obtainable with the usual centrally placed inocula. Samples of four cultures were taken daily over a period of 9 days, commencing 9 days after inoculation. The results given in Table VII show that an increase in dry weight of sporophores between the 12th and 13th days ($P = <0.01$) was accompanied by a reduction of similar magnitude in dry weight of mycelium ($P = <0.01$). Over this same period the total weight

remained virtually unchanged. Subsequently it increased at least as rapidly as before fruiting, although after the 15th day a reduction just short of acceptable significance coincided with the loss in weight ($P = <0.01$) by the sporophore crops through deliquescence. These results were confirmed in two similar experiments.

TABLE VIII

The Effects of Glucose-Alanine Ratio on Yields of Sporophore Tissue by Coprinus lagopus

Incubation period 15 days. Three replicates
Mean dry weights of sporophore crops (mg.)
Glucose (%)

		0	0.5	1.0	2.0	4.0
Alanine %	0	0.4	0	0	0	0
	0.05	0.4	12.6 (F)*	2.4	0	0
	0.1	0	12.6 (F)	26.1 (F)	1.7	0
	0.2	0	14.8 (S)	34.5 (F)	18.3	0
	0.4	0	6.5 (S)	16.9 (S)	1.8	0

Significant differences, calculated from analysis of data between

(i) 0.05–0.4% alanine and 0.5–2.0% glucose inclusive, $P = 0.05$, 10.9 mg.;
 $P = 0.01$, 14.8 mg.

(ii) 0.05–0.2% alanine and 0.5–2.0% glucose inclusive, $P = 0.05$, 4.1 mg.;
 $P = 0.01$, 5.6 mg. (see text for explanation).

* (F) indicates mature sporophores fertile; (S) indicates mature sporophores sterile.

TABLE IX

The Effect of Glucose-Alanine Ratio on the Age of Cultures of Coprinus lagopus at Onset of Fruiting

Incubation period 15 days. Three replicates
Mean age of cultures at onset of fruiting (days)
Glucose (%)

		0	0.5	1.0	2.0	4.0
Alanine %	0	11.3±0.9	—	—	—	—
	0.05	13.7±0.3	8.3±0.3	10.7±2.0	—	—
	0.1	—	7.0±0.6	9.0±0	11.3±0.9	—
	0.2	—	6.0±0	6.0±0	6.6±0.7	—
	0.4	—	6.0±0	5.7±0.3	6.0±0	—

(e) *The effects of carbon-nitrogen ratio on fruiting.* Cultures in triplicate were set up at each combination of 0, 0.05, 0.1, 0.2, and 0.4 per cent. alanine with 0, 0.5, 1, 2, and 4 per cent. glucose in Agar Medium A less Alanine and Glucose, and the media sterilized by steaming on three successive days. The results are given in Tables VIII and IX. As indicated in the footnote to Table VIII, the data were analysed statistically both with and without the 0.4 per cent. alanine treatments because the latter showed a considerable variation in the yields of sporophore tissue.

There was an interaction between glucose and alanine concentrations in

terms of yield of sporophores and the date at which fruiting began. Cultures at 1 and 2 per cent. glucose, which with low alanine produced little or no sporophore tissue by the end of the experiment, yielded well when alanine was increased. Probably this effect was largely attributable to the earlier fruiting in such cases. However, 0.4 per cent. alanine reduced yields with 0.5–2 per cent. glucose, even though fruiting commenced earliest, and interfered with individual sporophore development. A similar interference, in respect of fertility of the fruit-bodies, at 0.2 per cent. alanine and 0.5 per cent. glucose (Table VIII), was offset by increasing the glucose concentration to 1 per cent.

Of the two treatments with no glucose in which fruiting commenced, all

TABLE X
The Effects of Different Thiamin Concentrations in Agar Medium A on Growth and Fruiting of Coprinus lagopus

Thiamin concentration ($\mu\text{g.}/1,000 \text{ ml.}$)	Incubation period 13 days. Four replicates Mean dry weight (mg.)			Days to fruit
	Mycelium	Sporophores	Total*	
0	44.8	0	44.8	—
1	50.8	0	50.8	—
10	55.4	0 (+)	55.4	12.0–> 13
50	26.1	31.3 ± 2.3	57.4	9.0
250	27.7	30.2 ± 2.2	57.9	9.5
500	28.8	31.7 ± 0.9	60.5	10.0
5,000	35.4	29.5 ± 1.3	65.1	9.5

* Treatment effects not significant.

(+) Indicates primordia had just appeared in some replicates but were of negligible dry weight.

replicates with no alanine produced one or two extremely small sporophores similar to the 'dwarf fruit-bodies' of Buller (1922); all replicates with 0.05 per cent. alanine produced very small primordia only.

(f) *The effect of different thiamin concentrations.* Four cultures were set up at each of the following concentrations of thiamin hydrochloride in Agar Medium A less Thiamin, namely 0, 1, 10, 50, 250, 500, and 5,000 $\mu\text{g.}$ per 1,000 ml. of medium. The media were prepared with K_2HPO_4 in one solution and the rest of the nutrients plus the appropriate quantities of thiamin in another. After autoclaving, the two solutions were mixed aseptically and inoculated.

Although the treatment effects on total yield of fungus were not quite significant at $P = 0.05$ (Table X), the density of aerial mycelium as assessed visually was clearly decreased by reducing the thiamin concentration below 10 $\mu\text{g.}$ per 1,000 ml. of medium. Fruiting showed a very marked response. At 10 $\mu\text{g.}$ per 1,000 ml., primordia formed in three of the four replicates, but their dry weight was negligible; all treatments above this concentration of thiamin fruited equally well but all below it remained sterile. Since a total deficiency for thiamin was confirmed in a preliminary experiment on liquid

media, it seems probable that the relatively abundant vegetative growth in media to which no thiamin had been added was due to thiamin in the agar itself. The quantity was, however, insufficient to support fruiting.

(g) *The effects of different quantities of substrate.* Voderberg's (1949) report that a certain minimal quantity of mycelium was required for the fruiting of *Coprinus lagopus* was investigated in the following experiment. Discs of Agar Medium A of the same thickness were prepared of various diameters from 0.75 to 5.0 cm. These were made by pouring 20 ml. of sterilized medium into Petri dishes, each 90 ± 1 mm. in diameter with a flat base. Discs of appropriate size were then removed aseptically and several of various sizes placed in

TABLE XI

The Influence of Quantity of Substrate upon Yields of Vegetative Mycelium and Sporophores from Mature Cultures of Coprinus lagopus

	Diameter of disc of agar (cm.)								
	0.75	1.0	1.25	1.75	2.0	2.4	3.0	4.0	5.0
D.W. myc. (mg.)	0.1	0.2	0.2	0.5	0.8	1.6	1.9	3.6	4.9
	± 0.02	± 0.08	± 0.03	± 0.03	± 0.03	± 0.11	± 0.09	± 0.14	± 0.35
D.W. sporo. (mg.)	0.2	0.8	0.8	1.8	2.2	3.1	4.6	8.9	13.6
	± 0.02	± 0.16	± 0.08	± 0.14	± 0.07	± 0.21	± 0.31	± 0.58	± 1.25
myc./cm. ² (mg.)	0.32	0.29	0.19	0.22	0.25	0.34	0.27	0.28	0.25
sporo./cm. ² (mg.)	0.34	0.99	0.65	0.76	0.69	0.68	0.65	0.71	0.69
Days to maturity:									
mean:	10.7	10.4	10.4	11.7	11.5	12.0	12.5	12.9	12.8
range:	10-11	9-11	9-11	11-12	11-12	12	12-13	12-13	12-13
No. mature sporophores:									
mean:	1.7	1.5	1.2	1.5	1.6	2.3	1.9	2.8	3.2
range:	1-3	1-2	1-2	1-3	1-5	1-4	1-3	1-4	1-3
No. of replicates	(5)	(6)	(10)	(15)	(15)	(14)	(8)	(6)	(5)

each of a number of sterile Petri dishes and subsequently inoculated centrally. There were at least five replicates of each treatment; the numbers are given in parentheses at the foot of Table XI in which are presented the yields of vegetative mycelium and of sporophores *at the time of maturation of the first fruit-body* on each disc, and also the ages of the discs at the times of maturation.

It was found that discs of all sizes bore fructifications, although several of the smaller discs dried out before the sporophores matured. In all discs except those of the two smallest sizes, the dry weight of sporophore tissue was approximately directly proportional to their area and therefore to the volume of substrate. On all discs from 0.75 to 3.0 cm., the number of maturing sporophores was of the same order. Consequently, within these limits, the size of the maturing sporophores was related to the quantity of substrate. The fruit-bodies on the smaller discs were similar to Buller's 'dwarf-type'. On the 4.0 and 5.0 cm. discs the average number of mature sporophores was slightly higher, so that the ratio of volume of substrate to dry weight of individual mature sporophores became changed.

Fruit-bodies matured earlier on the smaller discs than on the larger. Since this could have arisen through either earlier initiation of sporophores or more rapid maturation of the smaller individuals, the previous experiment was

repeated, noting the ages of the discs at the time of the appearance of the first primordia and again at the maturation of the first sporophores. The results (Table XII) show that fruiting began earliest on the smaller discs and also that the period for maturation of the sporophores was virtually the same in all cases, even though the fruit-bodies were considerably larger on the bigger discs.

(h) *The effect of internal factors on the distribution of mature sporophores in cultures.* During these studies it was observed that although primordia of sporophores were usually more or less uniformly dispersed within an annular zone of the colony, enlarging and mature sporophores were usually very uneven in their distribution. Sometimes all arose in one-half of the culture.

TABLE XII

The Influence of Quantity of Substrate upon Onset of Fruiting and Maturation of Fruit-bodies of Coprinus lagopus

		Diameter of disc of agar (cm.)								
		0.75	1.0	1.25	1.75	2.0	2.4	3.0	4.0	5.0
Days to P.P.*	{ mean:	8.0	7.1	7.5	8.7	9.4	9.4	11.3	13.3	14.2
	{ range:	6-10	7-8	7-8	7-10	8-11	9-11	10-13	11-15	12-15
Days to M.S.*	{ mean:	12.0	11.1	11.6	13.2	13.5	13.8	15.7	>16	>16
	{ range:	12	11-12	11-13	11-14	12-15	13-15	15-17	16->17	16->17
Days to mature		4.0	4.0	4.1	4.5	4.1	4.4	4.4	—	—
Number of replicates		(6)	(12)	(13)	(13)	(14)	(10)	(7)	(10)	(6)

* P.P. = primordium production; M.S. = maturation of sporophores

These observations suggested that their distribution may have been governed by a nutritional mechanism similar to that proposed by Hein (1930b) in connexion with the abortion of supernumerary primordia in mushroom beds (Levine, 1922), namely that certain favourably placed young sporophores initiate a flow of nutritive materials in their direction that works to the disadvantage of less favoured individuals which thus fail to mature. By such a mechanism, slight irregularities in the initiation of primordia of *C. lagopus* would subsequently become greatly accentuated and manifested in the irregular distribution of the enlarging sporophores. According to this hypothesis, if the two halves of a culture were separated, then neither half would be able to draw on the intracellular reserves of the other and they should then produce similar yields of sporophores.

Accordingly two sets of five Petri dishes, each containing 20 ml. of Agar Medium A, were inoculated. Two similar plate cultures had been established the previous day in order to indicate when fruiting was imminent in the main series of cultures. On the 10th day, when the then 11 days old indicator cultures first showed primordia, the following treatments were applied to the two sets.

Series (i): a diameter of the colony was drawn across the base of each of the five Petri dishes, marking out two halves.

Series (ii): each culture was bisected with a sterile scalpel, one-half then being aseptically transferred to an empty sterile Petri dish.

All cultures were then incubated for a further 6 days, and the yields of sporophores and mycelium from each separated half and from the hitherto unseparated halves then determined. The results of this experiment and of a second conducted in a similar manner are presented in Table XIII.

The data reveal a great disparity in yields of sporophores from the two halves of intact cultures, whereas the separated halves of the other series bore almost equal yields. These results are thus in accord with the suggested mechanism. That distribution is governed by a *flow* of materials towards the

TABLE XIII
The Effect of Separation of the Halves of Colonies of Coprinus lagopus on the Distribution of Sporophore Material

	Five replicates (Yields in mg. dry weight)			
	Experiment I		Experiment II	
Mean yield of sporophore tissue from:	Unseparated halves	Separated halves	Unseparated halves	Separated halves
(i) more heavily fruiting halves	27.4 ±8.4	22.5 ±2.5	20.8 ±4.8	13.8 ±3.2
(ii) less heavily fruiting halves	3.6 ±1.8	18.8 ±2.5	3.5 ±4.8	12.3 ±3.4
Difference	23.8	3.7	17.3	1.5
Significance of diff. (<i>P</i>)	(0.03)	(0.3-0.4)	(0.04)	(0.7-0.8)
Mean yield of vegetative mycelium from:				
(i) more heavily fruiting halves	36.3 ±7.3	29.4 ±6.7	35.0 ±3.3	37.7 ±5.4
(ii) less heavily fruiting halves	41.4 ±4.4	31.3 ±4.5	41.2 ±3.2	35.0 ±4.2
Difference	5.1	1.9	6.2	2.7
Significance of diff. (<i>P</i>)	(0.5-0.6)	(0.8-0.9)	(0.2-0.3)	(0.7-0.8)

developing sporophores rather than general depletion of materials by fruiting, or even a form of inhibition between sporophores, is suggested by the following facts. Firstly, only a comparatively small number of primordia develop into mature fructifications. If it were simply a case of general depletion of the internal nutrient status of the mycelium by developing fruit-bodies one would expect that all of the primordia on a colony would develop into mature sporophores of all degrees of smallness, using those quantities of materials which are available, for it has been shown that exceedingly small amounts of mycelium can bear minute but nevertheless mature fruit-bodies. Secondly, a crop consisting of several sporophores will sometimes develop as a group, so that an inhibitory action is unlikely. The postulated *flow* of materials would permit favourably placed primordia to mature for example in a clump, yet would withhold nutrients from unfavourably situated primordia so that they had access to too low a level of nutrients to allow of further growth.

The data in Table XIII also show that the differences between the yields of vegetative mycelium from fellow halves in the two series of treatments were not significant. This suggests that the postulated flow of materials is spread

throughout the colony, impoverishing the mycelium more or less uniformly even though the sporophore crops may be most unevenly dispersed.

4. DISCUSSION

The isolate of *Coprinus lagopus* used in this work, like that of Voderberg (1948), was able to utilize cellulose. However, simpler carbohydrates too could be consumed and were able to support normal fruiting. Voderberg, on the other hand, was led to the conclusion that the organism, through becoming so closely adapted to its natural substrate, was no longer able to use carbohydrates other than cellulose. It would however seem certain that insoluble polysaccharides are enzymically hydrolysed to simpler soluble carbohydrates before they can enter the cells of a fungus. Such an hydrolysis was demonstrated above in the case of starch. The apparent need for cellulose may therefore have been caused by contaminating substances, perhaps essential growth factors, present in the materials which Voderberg used, viz. finely ground straw and blotting-paper.

The inhibiting effects of galactose and lactose are not easily explained. *C. lagopus* appeared to lack enzymes capable of hydrolysing lactose to glucose and galactose, for these products, at least in certain proportions, together supported good growth and gave a characteristic growth-form to the colony. Thus lactose must in itself have been inhibitory, and the fact that it retained its inhibitory effects in the presence of glucose was not surprising. However, galactose under these circumstances led to the more effective utilization of glucose, or itself became usable, since growth, although erratic, was on average far greater than with either sugar alone. Horr (1936) observed that with a strain of *Aspergillus niger* and with *Penicillium glaucum*, certain mixtures of galactose and glucose gave yields of mycelium greater than the total yield for similar quantities of these sugars used separately. The available information on these synergistic effects in fungi appears to be insufficient to satisfactorily explain the mechanism by which galactose and glucose together operate.

Zonation in fungi is not an uncommon phenomenon, and is usually related to some rhythmical change in the environment, such as fluctuating temperature or alternating light and dark (Hedgcock, 1906; Bisby, 1925; Hall, 1933). However, in a number of cases zonation has been reported in uniform external conditions (Hedgcock, 1906; Stevens and Hall, 1909; Hein, 1930a). The zoning of *C. lagopus* on glucose-galactose medium was of this type. Hein (1930a) described this sort of zonation in an unnamed sclerotium-forming fungus and suggested that the concentric zones were an expression of conditions in the substrate, or perhaps even in the hyphae themselves, which were analogous to those which cause the chemically produced phenomenon known as Liesegang's rings. It is interesting to note that the zonation described by Hein was dependent on the nature of the substrate, as was the case with *C. lagopus*, for it occurred only on certain media.

The reaction of *C. lagopus* to initial concentration of carbohydrate was similar to that of *Melanospora destruens* (Hawker, 1939). The response can

perhaps be more appropriately compared with that of *Collybia velutipes* (Plunkett, 1953). In this fungus the maximal yield of sporophores was attained at a lower initial carbohydrate (sucrose) concentration than that optimal for mycelial growth. Further increases in carbohydrate concentration caused an increasing number of replicates to fail to fruit, while the yield of those which did differed but little from the maximum. This was not wholly the case with *Coprinus lagopus*, for although the number of fruiting cultures declined with supra-optimal increments of glucose, the actual yields of sporophores by individual fruiting cultures were reduced because the sporophores failed to develop fully.

It is probable that the apparent antagonism sometimes to be seen between vegetative and reproductive growth (e.g. Table II) was the result of the different optimal carbohydrate concentrations for these two phases of growth. The reaction of a culture to an increased level of carbohydrate depended on the nutrient status of the medium relative to these optima prior to the addition being made.

The forms of nitrogen utilized included both organic and ammonium, but not nitrate. This result was intermediate between that of Leonian and Lilly (1938) who found only organic nitrogen could be used, and that of Voderberg (1948) who recorded that all sorts of nitrogen were usable, although organic sources were better. The differences are not irreconcilable. Leonian and Lilly conducted their experiments in media adjusted to pH 5.5. Results presented above show that the presence of calcium carbonate greatly enhanced the availability of ammonium-nitrogen, presumably by maintaining a favourable pH against the tendency for acid drift. Morton and Macmillan (1954) report that calcium carbonate operated in this fashion in connexion with assimilation of nitrogen from ammonium salts by *Scopulariopsis brevicaulis*. If then the pH drift in unbuffered media, initially at about pH 7.3, was sufficient to reduce the use of ammonium-nitrogen, that from an initial pH of 5.5 might virtually inhibit it. In the case of Voderberg's result, the basal medium used contained finely ground straw and a little sterilized yeast. This medium alone was capable of supporting some fruiting and presumably vegetative growth without additional nitrogenous material. It is therefore questionable whether the increase in fruiting obtained with potassium nitrate (6.4 to 7.2 on an arbitrary scale) really indicated its utilization by the organism, especially since fruiting does not always reflect total growth.

The effects of varied concentration of alanine have already been discussed in part, and the effects on fruit-body yield explained in terms of influence upon date of appearance of sporophore primordia and upon individual development. Plunkett (1953) recorded that the total sporophore yield of *Collybia velutipes* was reduced and individual development retarded by supra-optimal concentrations of asparagine, although ultimately some fruit-bodies matured; there was no effect of varied asparagine content upon the advent of the first primordia. *Coprinus lagopus* however produced primordia earlier upon alanine-rich media.

The peculiar response of the yield of vegetative mycelium to varied initial alanine concentration is explicable in terms of a transfer of materials from the vegetative mycelium into the developing fruit-bodies. The possibility that the fall in dry weight of the vegetative mycelium during fruiting (Table VII) was an artifact, due to an increased proportion of soluble materials which escaped in freeing the mycelium from the agar substrate, can be discounted, because, as noted above, the effect was also observed indirectly in investigating the effects of different concentrations of alanine in liquid culture media, when *true* dry weights of mycelia were determined. Although it has long been assumed that the production of fruit-bodies draws on materials from a large mass of mycelium (e.g. Buller, 1931, p. 152 et seq.) there appears to be no previously published account of this being demonstrated experimentally. It is possible that the process might be somewhat obscured in the case of *Hymenomycetes* in which sporophore production was accomplished more slowly than in *C. lagopus*, for then the transfer of material might not exceed the rate of formation of new tissue by the vegetative mycelium. It is probable that the sparser formation of aerial mycelium after fruiting commenced, which was noted above, was correlated with the depletion of materials within the colony by fruit-body production.

The concentration of thiamin which need be present for fruiting was greater than that necessary for mycelial growth. In this respect *C. lagopus* resembles *Ceratostomella fimbriata* (Barnett and Lilly, 1947).

The finding that *Coprinus lagopus* fruited on all quantities of medium tested, even as little as 0.14 ml. of Agar Medium A ($\equiv$ 0.75 cm. disc), is not necessarily at variance with Buller's view (1931) that sufficient vegetative mycelium to give rise to a fruit-body of a certain minimal size is a prerequisite of fruiting in the larger fungi, for the minimal size with *C. lagopus* must be very small indeed (less than approximately 0.2 mg. dry weight). Since there was an enormous variation in size of the fruit-bodies, it was unlikely that initiation of fruiting could be governed by an internal gross nutritional mechanism alone, for whereas on very small quantities of medium, approximately 0.3 mg. of fungus could give rise to mature sporophores, on 20 ml. of medium, far more than 0.3 mg. of mycelium was formed in the course of 2 or 3 days, yet usually about 10 days elapsed before primordia appeared. There was therefore a period of vegetative growth during which time either specific fruiting substances were formed, or conditions in the medium were rendered favourable, or both.

Relating these results to Klebs' (1900) views on fruiting in fungi (Coons, 1916) there is general accord except that there are instances where *C. lagopus* apparently behaved in a fashion inconsistent with his principle that conditions favouring reproduction are always more or less unfavourable for vegetative growth. Since fruiting removed intracellular materials from the vegetative mycelium it could not be determined by measurements of weight whether vegetative growth at fruiting became reduced or not. However the data on growth in terms of dry weight (Table VII) show that vegetative growth con-

tinued after the production of the first crop of sporophores at a rate comparable to that formerly held. Text-fig. 3 shows that the onset of fruiting did not necessarily coincide with a reduction in the radial growth rate of the colony. Over a limited range, increases in glucose concentration were accompanied by increases in both vegetative growth and sporophore yield (Table III) and similarly Table II shows that the addition of 0.5 per cent. glucose to the basal medium increased both growth and fruiting. Plunkett (1953) reported that the behaviour of *Collybia velutipes* was likewise not always consistent with this principle of Klebs.

5. SUMMARY

Coprinus lagopus produces fruit-bodies in culture on liquid and solid media containing glucose, dl- α -alanine, mineral salts and thiamin. The best sources of carbon are carbohydrates. However, lactose and galactose inhibit growth, although with 0.5 per cent. glucose, galactose increases it.

Ammonium- and organic-nitrogen is utilized but apparently nitrate-nitrogen is not. Calcium carbonate enhanced the growth made with ammonium nitrogen, but reduced that with peptone.

The range of glucose concentrations permitting fruiting was narrower than that allowing vegetative growth and the optima for the two processes did not coincide. Increases in glucose concentration delayed fruiting.

Alanine concentration affected the date of fruiting and the yield and individual development of sporophores. There is an interaction effect on fruiting between glucose and alanine concentrations.

A higher concentration of thiamin was needed for sporulation than for vegetative growth.

Fruiting withdrew materials from the vegetative mycelium.

No evidence was found of a minimal quantity of substrate for fruiting to take place. At least within limits, yield and individual dry weight of sporophores were more or less directly proportional to the volume of substrate. Sporophores of diverse sizes all matured on average over a period of 4 days.

Evidence of an internal mechanism affecting distribution of mature sporophores is presented. It is probable that the basis of this effect is a flow of materials towards already developing sporophores.

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Differentiation and Stability in the Development of *Nicotiana rustica*

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With one figure in the Text

ABSTRACT

Stability, or homeostasis, is that property of the organism buffering it against small random fluctuations of the environment and accidents of development. A means of measuring, within the individual, the stability of expression of one foliar and two floral characters is given.

Data are presented from a set of diallel crosses among five varieties of *Nicotiana rustica*, derived from different populations and grown in two successive seasons. This allows stability to be examined over a number of genotypes in the parent and F_1 generations, in two differing environments.

The leaf characters show some change from node to node, i.e. manifesting a gradual differentiation, as well as random fluctuations which are taken as evidence of instability. Like stability, this differentiation rate varies from genotype to genotype and shows genetic control.

Some attempt is made to find to what extent the different phenomena are related.

INTRODUCTION

THERE is now no lack of evidence relating the stability of development to genotypic control. This concept is fully discussed by Lerner (1954), but may be briefly defined as the mechanisms of the individual which permit it to stabilize itself against accidents of development and fluctuations in the external environment. Lack of stability manifests itself as non-adaptive variation of the phenotypes produced by one genotype. The measurements of this variation which have been made in the past may be classified into three types:

- A. Variation between genetically like individuals grown in two different environments.
- B. Variation between genetically like individuals grown together.
- C. Variation between repetitive structures within single individuals.

Each of the measures presents its own problems of technique and interpretation accentuated by the fact that there is little evidence, as yet, of their consistency, even where simultaneous studies have been made.

Type A differences may derive from several causes, such as potence-environment interaction (Lewis, 1954), or the coming into play of different

genes in the two sets of circumstances. Furthermore, they may be interpreted in terms of the adaptability of the organism to its environment, so falling outside the definition of non-adaptive variation. Using the change of family means over years Jinks and Mather (1955) compared the stability of inbred parents of *Nicotiana rustica* with their F_1 's, finding that the latter were not significantly more stable. In respect of plant height, the variation of F_1 's was, in fact, greater than that of the parents. Their unpublished data further show that in two out of three characters which they examined, viz. the stability of plant height and of flowering time, there was evidence of genetical differences between the lines. In the same paper Jinks and Mather examine intra-plot variation, the type B measure of stability. Genetical variation was found in respect of the stability of flowering time only, out of four characters examined, but how this corresponds to the type A differences is not clear. In no case was the average stability of heterozygotes greater than that of inbreds.

It is possible that intra-plot variation may be influenced in some families more than in others by chance fertility changes in the experimental field. On the other hand, intra-plant variation, i.e. type C observations, can be related directly to accidents in the organism's development, that is to its internal stability, or at least to only the most local of environmental upsets. It must be regarded as the most efficient method of assessing the stability of development and has the advantage that it can be used in segregating generations. On the other hand, its use is limited to only those structures which are repetitive and few figures are available in the literature. These include two cases in *Drosophila melanogaster* (Mather, 1953; Tebb and Thoday, 1954), where the degree of asymmetry between left and right sides for the number of sternopleural bristles has been used. Amongst plants it has been noted in *Primula sinensis* (Mather, 1946) that floral characters vary between different flowers more in inbred parents than in their F_1 's. This applied equally to comparisons between flowers of the same plant and between plants of one family. Mr. M. Green (unpublished data), in this department, found that variation within plants of *N. rustica* did not differ significantly between homozygotes and heterozygotes.

In the present work on *N. rustica* it has been possible to use intra-plant variation to measure stability, but also these figures may be compared with measures of the A and B types. The stability of three different organs is examined side by side, together with changes associated with the normal development of the organism. *N. rustica* is accustomed both to selfing and cross pollination in nature, although the former seems to predominate. While it may retain the balance of an outbreeder therefore, homozygous genotypes must be well balanced also. Most of the published data concern comparisons in outbreeding species, between genotypes in the heterozygous state, which occurs in nature, and homozygotes which do not. The outstanding observation there has usually been the greater sensitivity of inbred parents to small environment fluctuations when compared with their heterozygous F_1 's.

MATERIALS AND METHODS

Five inbred lines of *Nicotiana* were used, Nos. 5, 7, 12, 14, and 41 of those listed by Mather and Vines (1952). They were originally derived from different populations and have a subsequent history of fifteen or more generations of inbreeding. A complete diallel set of selfs and F_1 and F_2 crosses was grown in 1953 and 1954. The experiment was grown in two adjacent blocks each consisting of the 20 F_2 and 20 F_1 crosses randomized in plots of five plants, and every selfed parental line represented by two such plots.

In both years the pistil lengths (i.e. distance from the base of the superior ovary to the stigma) and stamen length (measured from the longest stamen where there were any slight differences within the flower) were recorded from three flowers on every plant. The three measurements give a mean square for each plant, based upon two degrees of freedom, a function of which is used as a measure of the plant's stability for the expression of the character concerned. In addition during the second season lamina length and breadth at the widest part were recorded for three leaves on every plant, from which the leaf shape index (length/breadth) was calculated. The peculiarities of these measurements will be dealt with in a later section.

STABILITY OF THE FLORAL CHARACTERS

While a variance is a convenient measure of intra-plant variation, it will only be a legitimate measure of stability if each repetitive structure on the plant tends to equal size, or shape, to its fellows. Departures from such equality could be occasioned by two factors.

1. Time—flowers produced at different times might reflect the general ageing of the plant or some progressive effect of the season.
2. Position—flowers may alter in character according to their position on the plant, due to tissue differentiation.

An attempt was made to assess the effects of these two factors by selecting the three flowers differently in the two seasons. In 1953 they were all taken from the terminal inflorescence, while in 1954 the first was taken from the centre of the terminal inflorescence, the second from lower down this same inflorescence and the last from an axillary branch. These measurements were also made at weekly intervals for convenience, the 1953 data having shown that time had no effect since the 1st, 2nd, and 3rd flowers did not differ when compared over all the parental families. The question is easily put to the test by analysis of variance on stamen or pistil lengths. The 4 parental plots (2 in each block) provided 297 flowers as one plant was missing from variety 14. The 296 comparisons include 2 which compare the overall effect of the 3 different times or positions and 4 for the size differences between the 5 lines: 8 may be associated with the interaction of these two effects. Of the remainder, 94 are used to test the consistency of plants within each variety and 188 are concerned with various interactions involving times or positions,

but these are not subdivided. As time has no effect, the 1954 data test the effect of the position on the plant. The mean squares are given in Table I relating to pistil lengths but the situation does not differ essentially from that of the stamens.

The analysis shows position to have a definite effect on the measurements, but as the mean square for the varieties $\times$ positions interaction has a probability of 1 per cent. the effect is not constant throughout the five varieties used. The varieties also differ considerably in the size of their floral parts as the second item indicates. Thus in 1953 the intra-plant variance provides an

TABLE I

The Analysis of Variance of Pistil Lengths in the Parental Families: 1954 data

Item	Degrees of freedom	Mean Square	P
Positions	2	12.72	$< 0.1\%$
Between varieties	4	277.27	$< 0.1\%$
Varieties $\times$ positions	8	1.86	1%
Plants $\times$ varieties	94	0.96	..
Remainder	188	0.71	..

adequate means of measuring stability. In 1954 an average measure was arrived at for each plot by a simple analysis of variance. That component of variation which may be ascribed to differences between positions was subtracted from the pooled intra-plants sums of squares of the five individuals. The remaining sum of squares, divided by 8, gives a variance representing the within plant variation in that plot, independent of position effect.

Having found a valid method of assessing stability, an analysis of variance on the figures for each family will show the connexion between stability and genotype. Here, however, is one last difficulty; stability itself is expressed as variances which have a skewed distribution and so cannot legitimately be subjected to an analysis of variance. A logarithmic transformation will adequately correct this. The occasional plant with its three measurements equal has a variance of zero, the logarithm of which is not finite. Consequently in 1953 it was necessary to add a small constant to the within plant variance of each plant before taking logarithms, the transformation being $\text{Log}_{10} (\text{Var.} + 0.050)$. In 1954 as one pooled figure was provided by each plot and this was never zero simply taking $\log_{10}$ was possible. In both cases the transformed measures of stability were distributed approximately normally. The effect of this slight difference in transformation on the comparison between years will be noted later.

The plot means summed over the two blocks are given for the stability (transformed variances) of stamens and of pistils in the two seasons in Table II. The row (y_r) and column (y_c) totals are given in each diallel table, and also the array totals ($y_r + y_c$). This sum is the total of all entries giving information about the genotype of the r^{th} parent. It is orthogonal to the other

TABLE II

The Stability Measure of Both Stamen and Pistil Lengths for each Family in the two Seasons

(a) 1953

		Stamens ♂ parent				
		5	7	12	14	41
♀ parent	5	0.917	1.007	0.703	1.757	1.142
	7	1.152	1.062	0.903	0.790	1.389
	12	1.253	1.233	1.814	1.567	1.176
	14	1.303	1.253	1.264	0.241	1.785
	41	1.241	1.142	1.207	1.969	0.986
y _r		5.866	5.697	5.891	6.324	6.478
y _r + y _r		11.392	10.993	12.934	12.170	13.028

		Pistils ♂ parent				
		5	7	12	14	41
♀ parent	5	0.480	0.795	0.792	0.943	1.275
	7	0.935	1.050	1.204	1.164	1.121
	12	1.581	1.429	0.935	1.154	1.527
	14	0.996	0.654	1.316	1.735	1.607
	41	1.419	0.270	1.472	2.262	1.929
y _r		5.411	4.198	5.719	7.258	7.459
y _r + y _r		9.696	9.672	12.345	13.566	14.811

(b) 1954

		Stamens ♂ parent				
		5	7	12	14	41
♀ parent	5	1.113	1.130	0.740	1.435	1.516
	7	1.487	2.316	0.687	2.200	2.491
	12	0.854	1.382	1.491	1.149	2.155
	14	1.349	2.175	1.643	2.011	1.528
	41	1.741	1.552	1.718	1.400	1.786
y _r		6.544	8.555	6.279	8.195	9.476
y _r + y _r		12.478	17.736	13.310	16.901	17.673

		Pistils ♂ parent				
		5	7	12	14	41
♀ parent	5	1.453	1.896	1.100	1.183	0.985
	7	1.725	1.568	0.927	1.766	2.482
	12	1.251	0.936	1.693	1.120	2.176
	14	1.072	1.862	1.562	1.459	2.026
	41	1.367	1.769	1.732	1.733	1.789
y _r		6.868	8.031	7.014	7.261	9.458
y _r + y _r		13.485	16.499	14.190	15.242	17.848

parents as each of these contributes twice to the total, viz. as a non-recurrent female parent in the column and male parent in the row. It also includes r^{th} parent as a homozygote (twice), as well as its various heterozygotic combinations. Inspection of the diallel tables reveals that the row and column totals show some consistency over characters within single years. The 1953 array totals for the stability of both stamens and pistils are low, indicating more stable development, in lines 5 and 7, while lines 14 and 41 appear less stable. Line 12 is not so consistent but is not extreme in either case. In 1954 line 5 is again the most, and 14 and 41 the least, stable family with 12 intermediate while line 7 appears to be relatively less stable than in the first season.

Hayman (1954) has described an analysis of variance to detect the presence of various genetical phenomena using this type of data, and his notation is used throughout. Five main effects are examined, significance for which would show:

- a* Lines—the presence of heritable differences between the lines, the sum of squares being derived from the array totals.
- b*₁ Mean Dominance Deviation—an overall difference in the stability of the homozygous parents as compared to the F_1 's.
- b*₂ Further Dominance Deviation associated with the r^{th} parent—this in effect measures any heterogeneity of the parent- F_1 relationship over the five parents.
- b*₃ A Remainder Term, involving certain complex interactions to which no straightforward genetical meaning can be ascribed.
- c* Line Reciprocal Differences—any extra-nuclear inheritance due to one or more of the parents.
- d* Further Reciprocal Differences not associated with *c*.

The consistency over duplicate blocks can be estimated for all these main effects. In the present experiment since there is clearly a fair correspondence between the stability of stamens and pistils, a combined analysis was carried out which was subdivided into characters, years, and blocks. The main effects are summed over years and blocks, and it allows a large number of interactions to be taken out. Most of the year and block interactions proved to be homogeneous and accordingly are not shown separately, an exception being made in the case of the first order interaction of years and lines ($Y \times a$). The legitimacy of summing over characters, blocks, and years will be tested in each case by the corresponding interaction terms. The analysis of variance is set out in Table III. The total number of plot means should have been $8 \times 25 = 200$, but one plot was missing in the second season so that its pistil and stamen stability means were lost. The total degrees of freedom therefore number 197. The duplicate error derived from pooled block differences is based on 94 degrees of freedom ($2 \times 24 = 48$ in 1953 plus $2 \times 23 = 46$ in 1954). The sums of squares relating to block differences were calculated for each year and then summed as the two blocks did not occupy the same ground in the two seasons so that comparisons within seasons only are relevant.

Of the main effects, only the lines item has a significantly high mean square ($P < 0.1$ per cent). Thus there is clear evidence of variation between the five lines, which can only be related to gene differences affecting the stability of the two characters. Inheritance must be nuclear as neither of the reciprocal difference items c and d is significant. That the relative stability of the five lines is not consistent over the two years has been noted above, and this is reflected by a large $Y \times a$ mean square. It is significant only at the 5 per cent. level, but such a genotype-environment interaction is only to be expected

TABLE III

The Analysis of Variance of the Stability Measures of the Floral Characters

	Item	Degrees of freedom	Mean square	P
a	Lines	4	0.456370	$< 0.1\%$
b_1	Mean Dominance Deviation	1	0.001676	
b_2	Further " "	4	0.098712	
b_3	Remainder	5	0.055004	
c	Line Reciprocal Differences	4	0.074206	
d	Reciprocal Difference Remainder	6	0.103120	
Y	Years	1	1.510322	$< 0.1\%$
C	Characters (summed over Y)	2	0.001092	$1-2\%$
B	Blocks (summed over Y and C)	4	0.054473	
$Y \times a$		4	0.222911	$2-5\%$
$Y \times$ the other Main Effects		20	0.097000	
$C \times$ Main Effects		24	0.042311	5%
$Y \times C \times$ Main Effects		24	0.067111	
$B \times$ Main Effects		94	0.078598	
	(summed over Y and C)			

* Probabilities relating to subnormal mean squares.

and indeed it is remarkable that this change is not more marked considering the magnitude of the seasonal difference. While the level of stability clearly varies between the genotypes of the different lines, there is no evidence that greater stability is the prerogative of the heterozygous or homozygous condition, as inflation of b_1 or b_2 would show.

The mean square associated with the overall difference between the two years (Y) gives a variance of 19.216, which would suggest a very significant change but for the necessity of using different transformations on the data in the two seasons. In effect this adds a constant to each stability measure in the second season: this of course can in no way affect the comparisons within each year. What is more important still, as each family mean is affected by about the same amount, all the year interaction items (such as $Y \times a$ discussed above) are also independent of the change. On the other hand, the overall mean for 1954 is inflated, as will be the mean square for years. The extent of this effect was estimated approximately by calculating the average difference in the plot means occasioned by the change in transformation. Correcting the mean square for years accordingly reduces it by slightly more than

one-half, and the variance ratio to *c.* 9 (0.1 per cent. $<P < 1$ per cent.). Thus there is a genuine seasonal difference; plants in 1954 being on average less stable than in 1953. This accords with the two seasons, the summer of 1954 being unusually inclement and variable.

The items for overall difference in the stability of the two characters, *C* (1 per cent. $<P < 2$ per cent.), and *C* × main effects ($P \approx 5$ per cent.) are both subnormal. The implications of this will be discussed below, but it may be noted here that neither these nor the higher-order interactions involving *C* (*Y* × *C* × main effects, which has a slightly lower mean square than the duplicate error) contributed to the error sum of squares.

To sum up, the stability of two morphological characters in the flower

TABLE IV

A Comparison of Homozygous Parents and their Heterozygous F₁'s showing their Respective Stability Levels for the Expression of Three Morphological Characters

	1953		1954		Leaves
	Pistils	Stamens	Pistils	Stamens	
Mean parent	1.226	1.004	1.592	1.743	2.872
Mean F ₁	1.196	1.262	1.534	1.517	2.877

is found to be controlled by nuclear genes and quantitative differences exist between the five lines used in the experiment. As Table IV shows, there is no regular inequality between the heterozygous and homozygous conditions in respect of the stability of pistils, stamens, or indeed of the leaf character which is discussed below. This does not disagree with the postulate of Lerner (1954, chapter 12), which forms the basis for his thesis regarding cross fertilized organisms; 'that departure from the breeding system normal for the species leads to loss of buffering powers'. Since *Nicotiana rustica* may be cross- or self-pollinated in nature, heterozygotes would not be expected to differ much from homozygotes in their buffering, or stability, properties. Here, however, where genic control of stability is evident, this indicates absence of potency, or heterosis for greater (or lesser) stability, rather than any intrinsic effect of the heterozygous condition.

DIFFERENTIATION AND STABILITY IN LEAVES

In 1954 it was thought desirable to obtain stability data of some somatic character in addition to the flower observations. The only appropriate repetitive structures were the leaves. A simple measurement of leaf dimensions, however, does not provide observations the variance of which can be taken as a measure of stability. The leaves of *N. rustica* are borne alternately and decrease in size from the lowest, i.e. they do not tend to a common size. In the hope that the shape might remain constant despite this reduction in size, the length and breadth, at its widest part, of the lamina were recorded

from three leaves on each plant, and the leaf-shape index then found in each case. The inflorescence consists of somewhat irregularly branching cymes, the main one terminal below which others may arise in the axils of foliage leaves. The three leaves measured were those immediately below the terminal inflorescence, but some error may have been occasioned by the difficulty in fixing the 'top' leaf in some cases owing to the irregular branching of the inflorescence. No help is offered by the form of the leaf as this does not alter suddenly to a bract structure in the inflorescence.

Examination of the indices reveals that, in most cases, the lowest of the three is more ovate, giving a smaller index, and successive leaves are more lanceolate, giving a larger index, towards the inflorescence. This trend was more marked in some varieties than in others. Now the assumption upon which the intra-plant variance is taken as a basis for measuring stability is that the repetitive structures of the individual should tend to be equal. Graphically this means that the measurements when plotted against the position of the organ on the plant should give a straight line parallel to the abscissa. Lack of stability is then reflected in random fluctuations about the line, i.e. about the mean measurement. The leaf index does not satisfy this requirement—as was the case with the 1954 flower data. While the problem is the same, the leaf information differs in that the position of the organ can be measured, i.e. as the 1st, 2nd, or 3rd node. Leaf shape can therefore be plotted against position on the plant to give a sloping line. (This was not deemed possible in the flowers where there was no valid measure of position.) A greater slope indicates a rapid change in shape to a more lanceolate leaf immediately below the inflorescence, and a more gentle slope, little such change.

Although the line is not parallel with the abscissa, this seems no reason for supposing that fluctuations about it should be any less an indication of instability in the developmental pattern, than are fluctuations about a horizontal line, provided the relation is genuinely rectilinear. The three indices from each plant were therefore subjected to a regression analysis to provide (i) a regression coefficient, b , associated with one degree of freedom, and (ii) a remainder sum of squares also with a single degree of freedom. These respectively measure the rate of differentiation of shape over the three nodes considered, and the stability of the plant in respect to this developmental pattern. The stability measure used below is a logarithmic transformation of the remainder mean square as is the case with the flower-stability measure. But, just as the validity of the measure of flower stability depends on fluctuations being about a single mean figure, so the validity of the two leaf characters depends on the linear regression being a true representation of the changing leaf shape. Two tests are possible:

1. By measuring more than three leaves the linearity of the regression may be tested over a greater number of nodes, and
2. If the regression is curvilinear, over those nodes at which the measurements were taken, the mid point of the three will tend to be more often below the fitted straight line if the true curve is concave, or above if convex.

At the end of the season not many plants had five consecutive leaves remaining undamaged, but in a number of cases where so many measurements were possible the resultant graphs gave no indication that a linear regression was inadequate. The plants of the four parental plots of each variety were scored for 'convexity' or 'concavity' of the three points. The assumption that the linear regression is adequate would require equal numbers

TABLE V

The Differentiation and Stability Measures of the Leaves

Differentiation is measured by the regression coefficient of lamina shape on node. A large value denotes more rapid change of shape, towards a more lanceolet form in the case of positive values and a more ovate form in the negative instance. The stability measure, a transformed variance, is larger for more variable (i.e. unstable) families.

(a) Leaf Differentiation

		♂ parent				
		5	7	12	14	41
♀ parent	5	0.211	0.208	0.424	0.335	0.242
	7	0.070	-0.031	0.526	0.390	0.055
	12	0.554	0.192	0.593	0.452	0.510
	14	0.476	0.144	0.537	0.374	0.349
	41	0.197	0.123	0.137	0.288	0.308
y_r		1.508	0.636	2.217	1.839	1.464
$y_r + y_r$		2.928	1.646	4.518	3.719	2.517

(b) Leaf Stability

		♂ parent				
		5	7	12	14	41
♀ parent	5	2.353	2.160	2.457	3.033	3.303
	7	2.029	2.039	3.319	3.352	1.821
	12	3.726	3.041	3.579	3.583	2.105
	14	2.774	3.281	2.704	3.336	3.706
	41	3.207	1.667	2.894	3.379	3.051
y_r		14.089	12.188	14.953	16.683	13.986
$y_r + y_r$		27.395	24.748	30.987	32.484	28.184

to fall into the two classes. The x^2 testing this over all the parents and the heterogeneity x^2 for the five varieties both had a probability of about 50 per cent.: i.e. a linear regression of leaf-shape index on node is a satisfactory representation of the differentiation of leaf shape, just below the inflorescence. The variation of points about this regression line consequently provides a measure of developmental stability comparable with that used for the flower characters. The plot means, summed over the two blocks, for every parental and F_1 family, are given in Table V for both differentiation of the leaves and the stability of this pattern.

Looking firstly at parental values of the differentiation data (which lie along the diagonal of the diallel table), variety 12 is seen to have a rapid change in leaf shape while that of variety 7 remains almost constant over the nodes examined. The other three parents fall between these two. An analysis of variance of the type used for the stability data in the previous section shows that this character is inherited and gives some information about the genetical components (see Table VI). The error mean square against which other items are tested is provided by the pooled block interactions. These proved to be homogeneous and so are not shown separately.

TABLE VI
The Analysis of Variance of Leaf Differentiation Data

	Item	Degrees of freedom	Mean square	P
<i>a</i>	Lines	4	0.060895	≤0.1 %
<i>b</i> ₁	Mean Dominance Deviation	1	0.000757	
<i>b</i> ₂	Further „ „	4	0.004723	
<i>b</i> ₃	Remainder	5	0.001548	
<i>c</i>	Line Reciprocal Differences	4	0.004066	
<i>d</i>	Reciprocal Difference Remainder	6	0.013315	1-5 %
<i>B</i>	Blocks	1	0.000098	
<i>B</i> ×	Main Effects	22	0.004722	

The significance of the 'a' item gives clear evidence of genetical differences of a quantitative nature between the varieties. The other item showing some degree of significance concerns reciprocal differences. There is no question of one or more of the lines being associated with a maternal effect as the 'c' item is not inflated at all. Further examination of the ten reciprocal pairs of F₁ families shows that three individual crosses give rise to most of the inflation of the 'd' mean square. These are the F₁'s from varieties 7, 12, and 12, 41, both having significant reciprocal differences when tested individually, and, to a lesser extent, 7, 14 (5 per cent. < *P* < 10 per cent.). In the first and last of these the F₁ families each resemble the male parent more than the female one. This suggests that the effect is a chance one as genuine cytoplasmic inheritance should show up as a maternal effect. Furthermore an examination of the F₂ means from selfing all the F₁ families concerned shows no carry-over of the differences to the next generation. It seems reasonable therefore to suppose that the large mean square obtained is the result of chance errors, the occurrence of which is not surprising in view of the difficulties, recognized above, of measuring the character. The *b*₁ and *b*₂ items in the presence of significant genetical variation offer a means of detecting the presence of dominance. If there is a tendency for preponderance of alleles giving greater (or lesser) expression to be dominant, the F₁ means will tend to depart from the mid-parent value in some or all of the arrays. Such departures are reflected in the *b*₁ and *b*₂ items. In this analysis no such evidence of dominance is apparent. There remains the possibility that dominance may still

be present but associated sometimes with greater and in other cases with lower expression of the character. A means of detecting dominance even in these conditions, as Jinks (1954) has shown, is offered by the regression of Covariance of F_1 means, in each array, with the non-recurrent parent (W), on the Variance of the array (V). A significant regression is an indication of any departures from simple additivity of the genes. Where dominance is

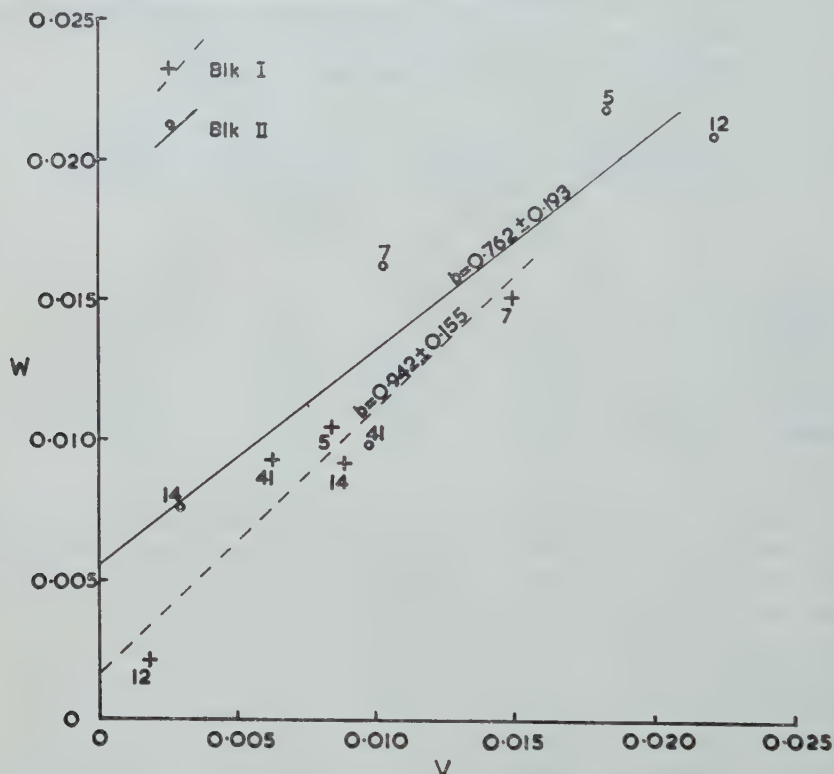


FIG. 1. The Regression of W on V for Leaf Differentiation.

present, and in the absence of genic interaction, the points tend to lie along a line of unit slope. The parents with the more dominant alleles fall near to the origin, and those with more recessive alleles farther from it. A significant regression is present in this case, the slope not significantly different from unity, so that the presence of dominance, but not of interaction, may be inferred (Fig. 1).

It can be seen that while three of the pairs of points agree well over blocks, those for varieties 7, 14, and 41, 12 appears to be the most dominant parent in Blk. I but the most recessive in Blk. II. Further, as an analysis of the regression shows, the mean of Blk. III is significantly greater than that of Blk. I. One contributory factor is evident; the mean for the parental plots of variety 12 in Blk. I is 0.215 and in Blk. II 0.378. This difference of more

than 0.150 is over double that in the case of any other parent and ten times the average increase in F_1 means from Blk. I to Blk. II. Since 12 is the parent with the largest mean, this will have two effects. Firstly, the variance of the parents will be greater in the second block, and secondly, as the increase in parent 12 is out of all proportion to the F_1 increase, offspring of 12 will be relatively more like their other parents, i.e. 12 itself will appear relatively more recessive in Block II. In fact, as we have seen, its change is from one extreme to the other. The estimate of the average dominance of the genes provided by the regression is $\sqrt{(H_1/D)}$, obtained from the two relationships:

$$\begin{aligned}\text{Variance of the parents} &= D + E_2 \\ \bar{W} - \bar{V} &= \frac{1}{4}D - \frac{1}{4}H_1 - \frac{4}{10}E_2.\end{aligned}$$

The E_2 or non-heritable component of the variance of the parental means derived from differences between duplicate plots, does not differ significantly from that of the F_1 's, given by the Blocks $\times$ reciprocal differences interaction term. These two are therefore pooled, giving $E_2 = 0.003830$. The estimates of average dominance for the two blocks then show:

$$\text{Block I} - D = 0.013169$$

$$H_1 = 0.002485$$

$$\sqrt{(H_1/D)} = 0.4344$$

$$\text{Block II} - D = 0.037529$$

$$H_1 = 0.021286$$

$$\sqrt{(H_1/D)} = 0.7531$$

Thus while there is some variation in the relative order of points, both blocks agree in showing partial dominance, the average dominance of genes being of the order of one-half to three-quarters. This does not necessarily mean that all the genes show, for instance, half dominance when the heterozygote falls mid-way between the mean parent and the homozygous dominant. It is an average figure for all the loci involved and could indicate, at the other extreme, that half the genes are fully dominant with the rest showing no dominance. It may be noted here that this type of analysis is also possible with the stability data in the flowers. It gives unambiguous evidence of the presence of dominance for that character also, but the error variation is such that a fuller analysis of the genetical control, along the lines given above, does not help to clarify the situation further.

Having examined some of the features of the genetical control of the variation in the differentiation of leaf shape, we can turn to the stability of this developmental pattern, which was also measured. The analysis of variance of this diallel table shows one significantly large item, that for lines, with 1 per cent. $< P < 5$ per cent., the error being provided by the pooled block interactions as before. The difference between the mean stability of all homozygotes versus heterozygotes (b_1) is subnormal, with $P \approx 5$ per cent. While a significance level of 5 per cent. is hardly a foundation for categorical statements, when taken alone, it is reasonable to postulate a genetic system controlling stability here, in the light of the situation in the flowers. There too the only significantly large item in the analysis within years (see Table III) is that for lines; and further the ' b_1 ' item is subnormal, although not

significantly so in that case. In this species where unbalance is not a result of enforced inbreeding we are able to recognize, in both flowers and leaves, that developmental stability results from the action of specific gene systems.

RELATIONSHIPS BETWEEN THE SYSTEMS

If the evidence is taken as showing that two gene systems are present which, in the leaves, affect the differentiation of shape and the stability of this pattern, we are left with the question of how these two are related. Now differentiation is a process in which initially small cytoplasmic differences are built upon in an ordered manner from one cell-generation to another in the growth of the organism. Thereby many different tissues are eventually built up in the mature organism, the cells of each being capable of multiplying true to their own type or in accordance with the pattern of further developmental change in that tissue, although the same nucleus is reproduced in all the cells. Such genetical implications of development have been discussed many times before (for instance, by Mather, 1948). By similar reasoning differences in structure of an unordered kind, i.e. instability, must be the end products of some more haphazard accumulation of cytoplasmic gradients giving differences between cells just as normal differentiation is founded upon ordered differences. Stability results from the successful control or suppression of such differences. This must hold whatever physiological basis is given to stability phenomena, such as the buffering effect of alternative synthetic pathways. One would expect that two phenomena, one depending on producing differences and the other upon their suppression, would not be independent but would tend to reinforce or cancel out, according to the phase of each in the individual. It may be imagined that the plant having a high differentiation figure because its leaf shape differentiates comparatively rapidly, would thus have the more readily a potential instability for the shape of each leaf. While it is not possible to see the two processes acting in development, the end products can be measured.

It is possible from the data above to examine the extent to which the phenotypic manifestation of higher differentiation rate goes hand in hand with higher variability of that same character. Pooling the two blocks, there are then two sets of measurements for all twenty-five genotypes possible when the five varieties are selfed and intercrossed (Table V). This gives a correlation coefficient for 23 degrees of freedom which is found to be $+0.5223$ giving $P \approx 1$ per cent. It is necessary to decide whether this implies that stability in this case is a side product of the differentiation. The correlation coefficient itself has a skewed distribution, but by means of the z transformation both upper and lower confidence limits can be fixed separately. The upper 1 per cent. confidence limit of the above estimate is found to be $c. 0.8$. A correlation coefficient of one-half indicates that the variance within these two foliar characters corresponds to the extent of one-quarter, and even at the upper 1 per cent. limit only 0.64 of the variance would be accounted for. In other words, the two systems overlap but are by no means coextensive.

Having genetical variation for both, a detailed examination should provide a unique study of change in development. Probably the flowers contain the more suitable structures for this purpose provided that their position on the plant can be determined exactly, allowing the differentiation rate to be measured as in leaf shape.

The other question of interest concerns the interrelations of the various measures of stability. This property has been associated with small fluctuations of the internal and external environments. It has been supposed in the past that no distinction between these two need be made and most of the published data measure stability by differences between like individuals sharing as near to a common environment as experimental techniques can provide. Here stability has been measured within the individual, but since plants were grown in plots of five the figures used can be compared to the stability measure provided by the within plot variance, the type of information used, for instance, by Jinks and Mather (1955). The correlation between the within plot variances and within plant variances for stamen height was calculated for the 1954 season. The stamen data were used for preference since, in that season, the stability differences between families were less marked in the pistils and therefore any correlations existing would be more likely to be obscured by the error variation. The correlation calculated over the twenty-five parent and F_1 families proved to be $+0.5826$, which is highly significant, 0.1 per cent. $<P < 1$ per cent. Considering that fertility gradients must affect the within plot variation, this figure may be taken as good evidence for regarding the two variances as substantially alternative measures of the same phenomenon. The constancy of expression of stamen and pistil lengths over the two seasons presents a different picture. When the change in family means over years for both organs is compared with the intra-plant measure of stability, no correspondence is found. ($r_{23} = +0.1274$). There is no evidence, in this case, that stability towards small local fluctuations is related to the plant's adjustment to gross environmental changes. This can hardly be universally so since the distinction between local and gross fluctuations is artificial, but it does serve to underline the difficulties in interpretation of this type of data as mentioned in the introduction.

The flowers provided two measures of stability within the plant based respectively on stamen and pistil measurements. The analysis of the data (Table III) shows, however, that the overall difference in stability between the two characters, the 'C' item, had a mean square significantly smaller than the error mean square 1 per cent. $<P < 2$ per cent., and the $C \times$ Main Effects interactions mean square was also low, $P \approx 5$ per cent. Clearly the stamens and pistils are providing two measures of the same thing: their level of stability is determined jointly, being under a single control, or at least they have many genes in common. This is not surprising, but it leads us to the more interesting question of whether there is a general control of stability throughout the plant. This would be reflected, for instance, in the correspondence between stability in the leaves and flowers. Correlations were

calculated on the 1954 diallel tables for flower stability (as measured from the stamens) and leaf stability, the two blocks being pooled. The straight correlation between the two is about -0.25 , but is, of course, influenced by the correlation noted above between leaf stability and differentiation. The partial correlation between flower stability and leaf stability independent of differentiation is very close to zero (-0.0314). The stability of the leaf differentiation is thus determined independently from that of the flowers. Now since the existence of genetical variation for stability between different natural populations of *Nicotiana* has been demonstrated, the level of stability presumably has selective significance—but that level need not be common to all organs. The above finding is not surprising therefore as a general level of stability existing throughout the organism would make the stability of all parts dependent on that of the most critical organ. As Mather (1953) says, 'we should expect the levels of stability achieved in respect of different characters to vary with the selective properties of the character'. He found evidence that in *Drosophila melanogaster* the bilateral symmetry for wing length is more rigidly controlled than for sternopleural chaetae number.

SUMMARY

A set of diallel crosses between five varieties of *Nicotiana rustica* was grown in two successive seasons. The stability of two floral characters, pistil and stamen lengths, was measured using the within plant variances estimated by recording from three flowers on every plant. Variation is found between the varieties in respect of stability, but the inbred parents as a whole do not differ from the mean F_1 . Analysis demonstrates nuclear inheritance of the differences and some genotype environment interaction across the two seasons.

The leaf-shape index is found to vary between nodes in two ways:

1. In a regular manner from node to node approaching a more lanceolate form, the rate of change being characteristic of each variety.
2. By fluctuations about this pattern which are interpreted as instability of development comparable to that examined in the flowers.

Both types of variability show the characteristics of quantitative inheritance, and it is suggested that as the two systems must act through a common cytoplasm during ontogeny, this may account for a positive correlation observed between their phenotypic expressions.

Within-plant variations of stamen lengths and pistil lengths are shown to reflect a common stability of the sexual organs. Since flower and leaf stability are found to be independent, there is some evidence that control is not general throughout the plant.

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Fructifications of an *Endogone* Species causing Endotrophic Mycorrhiza in Fruit Plants

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With Plates X-XIII and four Figures in the Text

ABSTRACT

The fructifications of a hitherto undescribed *Endogone* sp. have been found in organic connexion with mycorrhizal strawberry roots grown in pots in a greenhouse. By inoculating sterilized soil with these fructifications and with spores excised from them it has been possible to produce typical vesicular-arbuscular infections in strawberry, apple, and a number of other plants. These inoculation experiments constitute the first record of the establishment of endotrophic mycorrhizal infection in plants raised under aseptic conditions.

INTRODUCTION

THE systematic position of the fungi causing vesicular-arbuscular mycorrhizal infections has long been a matter of discussion. Fruit-bodies have been described by Peyronel (1924, 1937) who was able to trace organic connexion between mycorrhizal roots and the fructifications of *Endogone vesiculifera*, *E. fuegiana*, and one other unidentified *Endogone* species. The observation has, however, never been confirmed. Butler, who reviewed all the available evidence in 1939, concluded that the fungi concerned belonged to the Phycomycetes, and were probably closely related to the Endogonaceae. Three years ago fructifications of an *Endogone* species were found attached to mycorrhizal strawberry roots, and it was possible to demonstrate hyphal connexion between these fruit-bodies and the endophyte in the roots (Mosse, 1953). In the present paper a description is given of these fructifications, and the results are reported of inoculation experiments with the sporocarps and with spores excised from them.

THE FRUIT-BODIES

A fruit-body and its hyphal connexion with a strawberry root are shown in Fig. 1. The fruit-bodies, which are hypogeous, slightly translucent, brownish-black bodies, are approximately spherical and up to 1 mm. in diameter. They consist of a loosely interwoven mass of hyphae and finely divided soil particles with one or more spherical to pyriform spores embedded near the centre (Pl. X). There is no true peridial layer, but in sporocarps free from contaminating soil particles two definite regions can be distinguished (Pl. XI). The outer layer consists of slender, thin-walled, irregularly septate hyphae, 2 to 5 μ in diameter. Some of these project into the soil as

short hairs. Surrounding the spores, and often closely adpressed to them, is a region of coarse, thick-walled, densely staining hyphae, $7-10\ \mu$ in diameter. These appear to originate as branches from the spore-bearing mycelium and frequently arise just below the spore. Both types of hyphae show the characteristic wide-angled, more or less dichotomous branching typical of other *Endogone* species, and anastomoses leading to plexus-like enlargements are common (Fig. 2). Sporocarps may contain up to 32 (usually 2 to 6) spores,

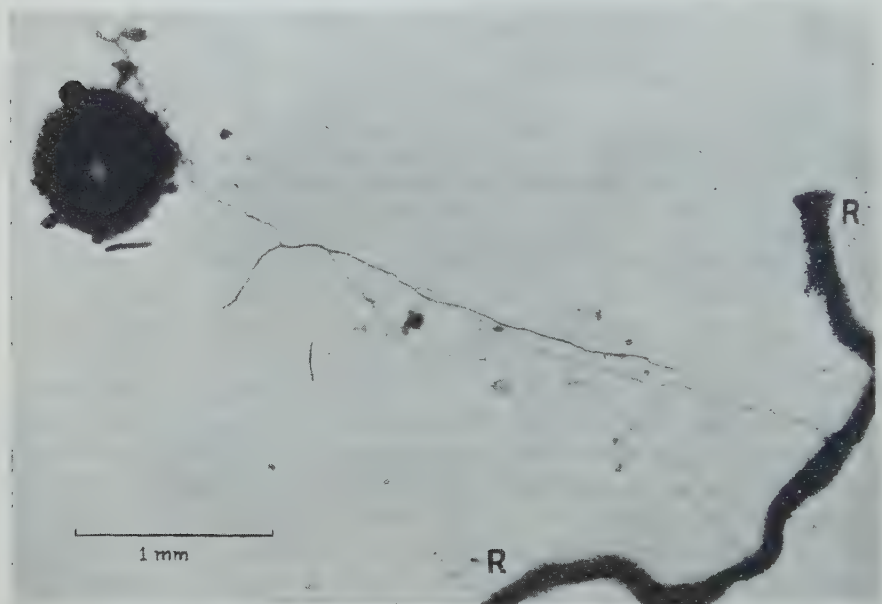


FIG. 1. Fruit-body and its hyphal connexion with a strawberry root (R).

$60-160-250\ \mu$ in diameter and generally, though not always, in the same stage of development. Young spores are thin-walled, hyaline, and have a strong affinity for cotton blue. As the spore matures a yellow endospore wall up to $7\ \mu$ thick, impervious to cotton blue and resistant to drying, is laid down internally, while the original spore wall loses its ability to take up stains, and develops into a thin, transparent, easily detachable envelope. The yellow endospore wall extends some distance into the hyphal stalk. In fully developed spores the spore contents are separated from the protoplasm of the subtending hypha by a continuation of the internal layers of the endospore wall across the hyphal stalk. Pl. XI shows the typical structure of the spore base at maturity. There is some doubt as to the correct interpretation of these structures and as to the nature of the spores. Hawker (1954) regards them as chlamydospores. Thaxter's (1922) criterion of a continuous endospore with spore contents separated from the cavity of their origin, suggests that their wall structure is more like that of zygosporcs, and in the development of their spore walls they show considerable similarity to the zygosporcs of *E. lactiflua* as described by Bucholtz

(1912). Although spores not infrequently have two or even three separate subtending hyphae (some typical spore attachments are shown in Fig. 3), definite evidence of gametangia has not been found, and it therefore seems improbable that these spores are the result of a sexual fusion. On the other hand, they differ from the chlamydospores borne freely on the extra-matrical



FIG. 2. Part of the hyphal matrix of a sporocarp showing the characteristic branching and anastomosis of the hyphae. (Tracing of projected image.)

mycelium in that the latter have open connexions with their subtending hyphae, and no clearly defined exospore wall. It is suggested that the resting spores in the sporocarps may be azygospores, and it is hoped that cytological investigation may clarify the matter. In some spores the thick endospore wall is traversed by many very fine fissures and occasionally spores are found with characteristic tooth-like projections growing from the spore wall towards the interior. In Pl. XI, 4, some of the projections are shown in detail, and a fine capillary opening running through them can be clearly seen. These projections bear some similarity to structures found by Thaxter (1922) in some spores of *Endogone macrocarpa* and to the *procès conique* described by Peyronel (1923). The spore contents consist either of a large central oil globule and a peripheral layer of protoplasm, of strongly vacuolated protoplasm,

or of a dense mass of even-sized fatty or protoplasmic bodies, sometimes interspersed with fine hyphae.

There is no doubt that the fructifications described above belong to the

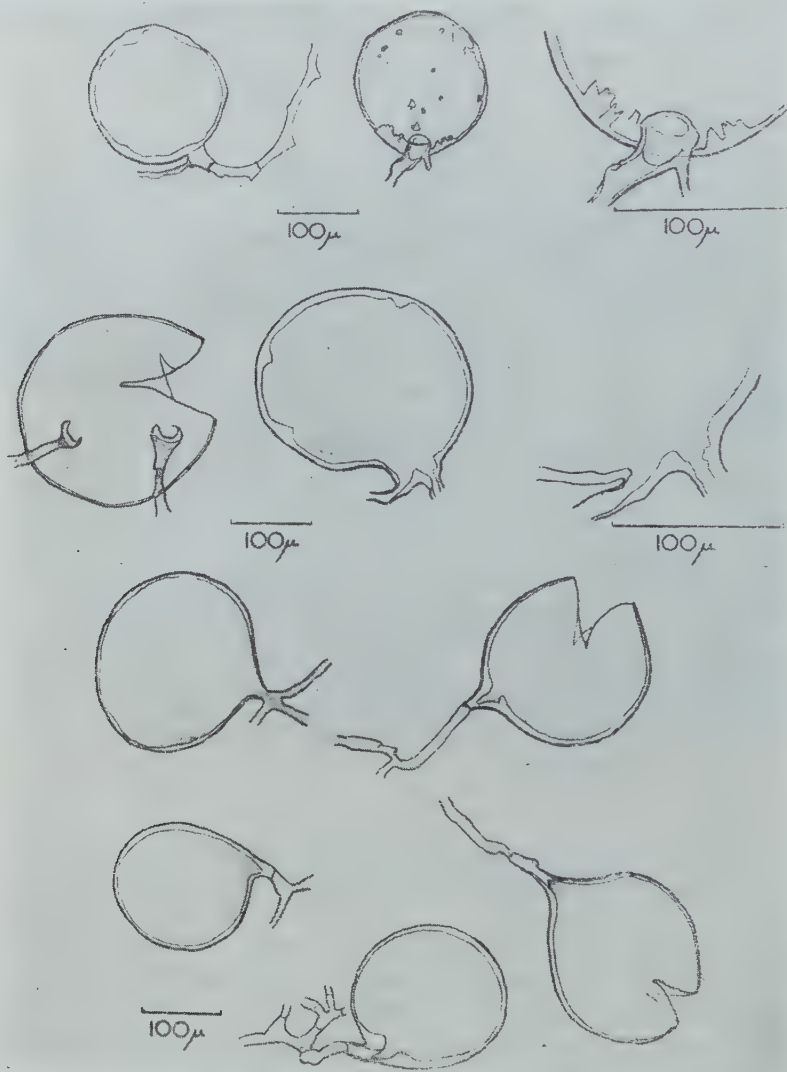


FIG. 3. Some typical spores and their hyphal attachments to the sporocarp matrix. (Tracing of projected image.)

genus *Endogone* as described by Link (1809) and accepted by Thaxter (1922). It is equally clear that the fructifications are different from those of the thirty-one named *Endogone* species described in Thaxter's monograph. This view is supported by Hawker (1954) who kindly examined some of the material. With a sporocarp size of 1 mm. or less the fruit-bodies are con-

siderably below the size range so far described for *Endogone* fructifications, the smallest known one being that of *Endogone incrassata* (2–5 mm. diameter) which bears no structural similarity to the species here described. In its very large spore size (up to 250μ diameter) the present species is more like *Endogone macrocarpa*, one of the three species occurring in Britain, and *Endogone tenebrosa* which it resembles in possessing a thin hyaline exospore wall. To judge from a somewhat incomplete description (Thaxter, 1922), *E. tenebrosa* differs from the present species in the thickness and colour of the spore wall, the nature of the spore contents, and suggested structure of the sporocarp. The chief distinguishing features between *E. macrocarpa* and the present species are the size and macroscopic appearance of the fruit-bodies, and the shape and wall structure of the spores.

Attempts are in progress to culture this fungus and it is intended to name it when more information is available regarding its life cycle and the development of its sporocarps. The above description is given in the hope that it may enable others to find similar fructifications associated with the mycorrhizal roots of other plant species. This would be of special interest, as the fructifications have not so far been found under field conditions, but only in association with plants grown in pots in a greenhouse.

METHODS

Before considering the inoculation experiments it is necessary to discuss shortly certain investigations concerning (a) the raising of uninfected test plants with normal root systems, and (b) the methods of introducing the inoculum. The fifteen inoculation experiments to be described were conducted over a period of three years, and during this period certain improvements in technique were developed.

A. The raising of test plants

Strawberry runners var. Royal Sovereign and seedlings of *Fragaria vesca* were used as test plants. They were raised under conditions varying from partial to complete sterility, i.e. from commercially steam sterilized soil in porous flower pots to autoclaved and chemically sterilized soil and nutrient agar in plugged test-tubes. Commercial steam sterilization did not completely eliminate mycorrhizal infection, although plants in inoculated treatments always became infected more quickly and to a greater extent. The method finally adopted for the open pot experiments was to autoclave pots containing a coarsely sieved loam and sand mixture with a 1-in. surface layer of pure sand, at 15 lb. pressure for 30 mins. The covered-up pots were stored for at least 6 weeks, and were then soaked in tap water and stood individually on sterilized bricks placed on a bench in an insect-proof greenhouse with roof ventilation. Young runners, not previously in contact with the soil and therefore free from mycorrhizal infection, were then rooted into these pots. Fresh tap water was used for watering.

For experiments in plugged test-tubes seedlings were used, and four different media were tried: (i) autoclaved soil, (ii) soil sterilized with propylene oxide, (iii) autoclaved sand irrigated with a culture solution, and (iv) nutrient agar.

(i) *Autoclaved soil.* Growth was very variable in this medium. Some seedlings grew quite well, while others were stunted, had yellow leaves, and sometimes died outright. Intermittent sterilization, by standing tubes in boiling water for 30 min. on three successive days, was also tried but resulted in uniformly stunted and unhealthy plants. A uniform stand of reasonably good seedlings could be obtained by adding activated powdered charcoal to the soil (Turner, 1955). 15 ml. of a fairly dry mixture of sieved loam and sand (2:1)+5 per cent. powdered charcoal (by volume) were placed into plugged test-tubes and autoclaved at 15 lb. pressure for 30 min. Seeds could be sown in this almost immediately and made satisfactory growth.

(ii) *Soil sterilized with propylene oxide.* 1 ml. of propylene oxide was added to 15 c.c. of a fairly dry loam and sand mixture and left to stand in plugged test-tubes for at least 4 weeks until all smell of the propylene oxide had disappeared. The soil was then watered with sterile distilled water, and seeds were sown in it. Sterilization by means of propylene oxide is less effective than autoclaving. Since, however, soil sterilized with propylene oxide yielded a higher proportion of successful inoculations, various modifications of the propylene oxide treatment are now being tried in the hope of finding one that will give complete sterility.

(iii) *Irrigated sand.* Autoclaved sand irrigated with two different culture solutions was tried. Seedlings grew well at first, but after 6 weeks were smaller than those in autoclaved soil and had rather abnormally branched root-systems. As they also required more frequent watering no further test plants were raised in this medium.

(iv) *Nutrient agar.* Of the several different media tried the following gave the best results:

	gm./litre of distilled water	Trace elements	p/p/m
$(\text{NH}_4)_2\text{SO}_4$. . .	0.26	boric acid	0.5
K_2HPO_4 . . .	0.2	zinc sulphate	0.06
$\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$. . .	0.8	copper sulphate	0.06
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$. . .	0.6	manganese sulphate	0.25
FeSO_4	0.017		
	(freshly made up)		
Agar	12.0		
	pH adjusted to 6.3		

All test-tubes were plunged in a damp sand pit erected on a greenhouse bench so that the leaves could have the maximum amount of light while the roots were kept in the dark and prevented from overheating. It was found that additional lighting was necessary for growth during the winter months.

It is noteworthy that a soil extract medium, made by shaking up soil in

water and autoclaving the filtered extract after addition of 2 per cent. agar, produced extremely poor seedling growth. The roots were stunted, turned blackish-brown, and the plants died after a few weeks.

B. Methods of introducing the inoculum

Whole sporocarps or spores dissected from them were used as inoculum. Sporocarps were either used fresh or were surface sterilized for 5 min. in 0.1 per cent. mercuric chloride+0.01 per cent. dioctyl sodium sulpho-succinate (aerosol O.T.). In either case a number of contaminants were probably introduced with the sporocarps since their loose structure and the deeply embedded organic particles make it impossible to sterilize them adequately without killing the fungus.

For more critical experiments individual spores were dissected from sporocarps, freed from the remains of the thin exospore wall, and sterilized for 3 min. in 0.1 per cent. mercuric chloride+aerosol O.T. Later mercuric chloride was replaced by a freshly prepared solution of 2 per cent. chloramine T used for 4 min.

Spores were washed repeatedly in sterile distilled water, and were then placed in the sterilized soil in test-tubes by means of a wire loop. At first spores were sterilized in bulk in an autoclaved glass box with a filter-paper bottom, allowing sterilant and rinsing water to be drained from the box. Later spores were sterilized individually by moving them on a wire loop through a series of dishes containing sterilant and rinsing water. Sterility of the spores was tested by placing them on nutrient agar and in hanging drops of sterile water. Occasionally bacterial contamination developed. In addition a very fine mycelium ($1-3\ \mu$ in width) developed from a proportion of the spores, emerging all over the spore surface and occasionally even through the old spore attachment. Isolations of this organism were made from some fifty individual spores, and all the isolates proved to be identical, but it has not so far been identified. When used as inoculum it failed to produce a single mycorrhizal infection in seventy separate inoculation tests, even under the least stringent conditions under which sporocarp inoculations regularly produced 100 per cent. infection of test plants. It is now believed that this fine mycelium may belong to a parasite, possibly an actinomycete, which occurs commonly within the *Endogone* spores and remains unaffected by surface sterilization. The regular occurrence of a parasite in the spores of some *Endogone* species has been reported by Thaxter (1922), and there is evidence that in this species also a considerable number of the spores are so infected. No other contaminants developed from test spores after sterilization.

SPOROCARP INOCULATIONS

Table I shows, in chronological order, the results of seven inoculation experiments carried out between 1951 and 1953. Experiment III has since been repeated three times with identical results. The survival of some

TABLE I
Results of Inoculation Experiments with Sporocarps

Expt. No.	Test plant and medium	No. of sporocarps per pot or tube	Inoculated in	Examined after	No. of roots examined per plant	No. of plants that developed mycorrhiza	
						Inoculated	Control
I } II }	Strawberry runners (var. Royal Sovereign) in steam sterilized soil in open pots	20 unsterilized 30 unsterilized	Aug. April	34 weeks 12 weeks	6 2	2/2 16/16	0/2 0/8 re-examined in Sept. 6/8
III*	Strawberry runners (var. Royal Sovereign) in autoclaved soil in open pots	30 sterilized	Aug.	21 weeks	8	6/6	0/6
IV } V } VI } VII }	Seedlings of <i>Fragaria vesca</i> in autoclaved soil in plugged test-tubes	5 sterilized 5 sterilized 5 unsterilized 15 sterilized	May May Sept.	9 weeks 11 weeks 25 weeks	4 { 4 4 4	2/5 1/4 1/4 3/5	0/5 0/4 0/4 0/5

* The same result has been obtained in three subsequent experiments under similar conditions.

mycorrhizal fungi after steam sterilization has already been mentioned. It is shown by the results of experiment II and also of experiment VIII (Table II). With the exception of these two experiments control plants in uninoculated treatments always remained free from mycorrhiza.

It was anticipated that it might prove difficult to determine whether a plant had become mycorrhizal or not without examining large numbers of roots. In fact this was not so. A sporadic infection of the root system was unusual, and if a plant became mycorrhizal at all, the whole of its root system was generally affected. When the roots had been carefully washed, plants which had become mycorrhizal could be picked out almost at a glance by the extra-matrical mycelium adhering to their roots, and by the very fine soil particles attached to the mycelium. Fig. 4 shows a photograph of the roots of a successfully inoculated seedling, an inoculated seedling which did not become mycorrhizal and an uninoculated control. Under a dissecting microscope the typical coarse hyphae of the endophyte, bearing vesicle-like spores, could be clearly seen entering the roots. New sporocarps were also formed abundantly in inoculated treatments. The procedure followed was to pick out, under the dissecting microscope, roots into which hyphae were penetrating, to section these roots, and to examine them microscopically, after mounting in polyvinyl lactophenol with a trace of cotton blue (Metcalf and Richardson, 1949). An equal number of the most likely looking roots from control plants were also selected for microscopic examination.

The most detailed examination of the root-system was made in experiment III. Out of 48 roots selected under the dissecting microscope as having hyphae penetrating into them, 47 roots showed typical mycorrhizal infections on sectioning. No hyphae could be seen entering the roots of control plants, and of the 48 most likely looking roots which were sectioned none were mycorrhizal. Although it seemed clear that the presence of the extra-matrical mycelium could be taken as satisfactory evidence of infection, it still seemed doubtful whether its absence could be taken as sufficient proof of non-infection. It was therefore decided to continue sectioning some roots of each plant. One hundred and seventy roots belonging to 35 uninfected plants were examined in this way. None of the 35 root systems were associated with the extra-matrical mycelium, and none of the 170 roots examined were mycorrhizal. It was therefore concluded that, under the conditions of the inoculation experiments, the presence or absence of the typical spore-bearing extra-matrical mycelium could be used as a criterion of infection. The mycelium can be recognized with reasonable certainty at magnifications obtainable with a good stereoscopic dissecting microscope (at approx. $\times 70$). It was possible to examine in this way a far greater proportion of the root-system than could be examined by sectioning individual rootlets.

The mycorrhizal infection obtained in inoculated treatments resembled in every respect infections which occur in strawberry roots grown under natural conditions in the field. Arbuscules and vesicles were formed in the usual way and the inter-cellular hyphae had the characteristic appearance of the

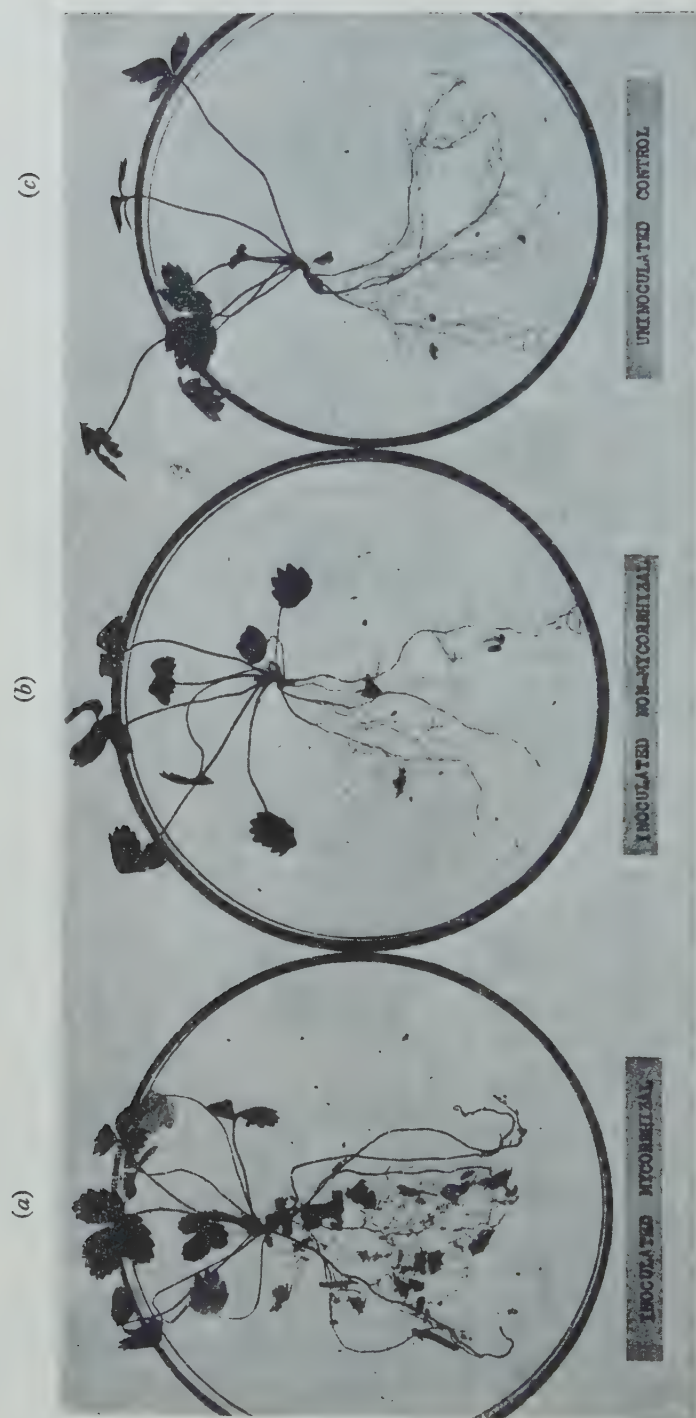


FIG. 4. Seedlings of *Fragaria vesca* raised in plugged test-tubes in compost (loam, peat, and sand) sterilized with propylene oxide; (a) and (b) inoculated with sterilized spores; (c) uninoculated control. The mycorrhizal seedling (a) is easily distinguished from the non-mycorrhizal seedlings (b) and (c) by the finely divided organic matter adhering to its roots.

endophyte. New sporocarps were produced abundantly under open pot conditions.

The results shown in Table I indicate that the proportion of successful inoculations depended largely on the experimental conditions. In open pot experiments all the inoculated plants became mycorrhizal. In plugged test-tubes, on the other hand, only 25-60 per cent. of the inoculated treatments

TABLE II
Results of Inoculation Experiments with Spores

Expt. No.	<i>Fragaria vesca</i> seedlings raised in:	No. of spores per tube	Inoculated in	Examined after	No. of plants that developed mycorrhiza	
					Inoculated	Control
VIII	Loam + sand (steam sterilized) + peat (fresh)	5 sterilized HgCl ₂ + 10 fresh	May	12 weeks	3/4	1/4
IX	Loam + peat + sand sterilized propylene oxide.	5 sterilized HgCl ₂	May	10 weeks	4/8	0/7
X	Loam + sand autoclaved	5 sterilized HgCl ₂	May	7 weeks	1/5	0/5
XI	Nutrient agar	5 sterilized HgCl ₂	April	52 weeks	0/6 spores did not germinate	0/6
XII	Loam + sand autoclaved	15 sterilized HgCl ₂	Sept.	30 weeks	2/6	0/6
		25 unsterilized from five different sporocarps	Sept.	21 weeks	3/5	
XIII	Loam + sand + peat + charcoal sterilized propylene oxide	20 sterilized HgCl ₂ (Source 1)	August	21 weeks	5/6	0/7
		20-30 ster. (Source 1)			4/4	
		20-30 unsterilized from five different sporocarps (Source 2)			0/2	
XIV	Loam + peat + sand sterilized propylene oxide	20-30 sterilized HgCl ₂	Sept.	20 weeks	1/6	0/6
		20-30 sterilized HgCl ₂			0/6	
XV	Loam + sand sterilized propylene oxide	5 sterilized chloramine T	Sept.	20 weeks	5/6	0/6

became infected. Comparison of experiment III with experiments IV to VII shows that high infectivity may be related to open pot conditions.

Mycorrhizal infection could be induced in *Fr. vesca* with sporocarps derived from the strawberry variety Royal Sovereign. Typical infections were also produced in apple, wheat, grasses, tomato and lettuce grown in autoclaved soil in open pots, and inoculated with sporocarps from strawberries. Tomato and lettuce roots became infected occasionally, while heavy infections were produced in apple, wheat, and grasses. The infections were in every respect similar to those occurring under natural conditions. Pl. XII, 5 shows the mycorrhizal structure of an apple rootlet from an inoculated treatment.

SPORE INOCULATIONS

Inoculation experiments in which excised spores were used as a source of the fungus are set out in chronological order in Table II. In all these

experiments seedlings of *Fragaria vesca* grown under sterile conditions were used as test plants.

Ignoring experiment VIII in which steam sterilized soil was used, none of the 43 controls became mycorrhizal, while typical mycorrhizal infection developed in 25 out of 60 inoculated treatments. Sterilized spores were used as inoculum in 18 of the 25 successful inoculations. Infections were in every way similar to those occurring under natural conditions and some typical arbuscules and vesicles which developed in the inoculated plants are shown in Pl. XII, 6. The extra-matrical mycelium was very strongly developed, and new sporocarps were also found in a few of the inoculated treatments.

The success of individual inoculation experiments varied from 0 to 100 per cent. Consideration of the results suggested that the most important single factor was some inherent condition of the spores themselves, and this idea has been strengthened by further inoculation experiments during 1954. In these mycorrhizal infections were produced in 11 out of 50 inoculated treatments. The proportion of successful inoculations is relatively low because the number of spores was deliberately reduced to 1-4 per tube, and different experimental conditions were tried. A source of spores was found which, under favourable conditions, produced from 50 to 90 per cent. germination, with a thick strongly translucent germ tube arising from the old hyphal attachment of the spore. From this developed a coarse, sparsely branched mycelium very similar to the extra-matrical mycelium of the endophyte, and bearing like it vesicle-like spores with open connexions. A germinated spore and the mycelium arising from it are shown in Pl. XIII, 7. Conditions favouring the growth of this mycelium are still being studied. It is therefore not intended to discuss them further at present, except to record that inoculation with individual germinated spores produced typical mycorrhizal infection in strawberry seedlings growing in autoclaved soil in plugged test-tubes. On the other hand, no mycorrhiza developed and hyphal growth failed to continue when germinated spores were placed on the roots of strawberry plants growing in nutrient agar.

The fungus has not so far been reisolated from inoculated plants. Limited growth and the production of new vesicle-like spores have been obtained by planting out whole seedlings or substantial parts of the root system of inoculated plants in 2 per cent. plain agar. Under these conditions some vigorous new hyphae emerge, and continue growing for about 10 days. Growth then stops, the hyphae lose their translucency, their walls thicken and become yellow, and frequently septa develop as the protoplasm recedes from the hyphal tip. The new hyphae generally originate from the root surface, but occasionally they also arise from broken hyphae of the extra-matrical mycelium or as new branches from this.

DISCUSSION

The purpose of the present paper has been two-fold: (1) to describe the fructifications of an *Endogone* sp. found associated with mycorrhizal straw-

berry roots, and (2) to give an account of experiments in which typical vesicular-arbuscular mycorrhizal infections were established by inoculation with sporocarps and spores of this fungus. It remains to consider how far the available evidence proves that this *Endogone* sp. causes vesicular-arbuscular infection in plants.

Positive evidence rests (1) on the repeatedly observed organic continuity between sporocarps and the vesicular-arbuscular infection in mycorrhizal strawberry roots (Mosse, 1953), and (2) on the inoculation experiments. Sporocarp inoculation of autoclaved soil in open pots regularly and repeatedly produced typical mycorrhizal infection in the roots of strawberry runners grown in it. Chance infection of controls occurred very occasionally, but it was rare and always developed more slowly. Since sporocarps, whether sterilized or not, are not a pure source of inoculum, it is arguable that the infections produced were due to some contaminating organism introduced with the sporocarps. This view is, however, discounted by the successful inoculations with sterilized spores in plugged test-tubes where the risk of such contamination would be small. Spores incubated after sterilization grew only one type of very fine mycelium, which when tested was repeatedly found ineffective as a mycorrhiza former. In all the experiments the soil was sterilized with propylene oxide or by autoclaving on a single occasion. While neither method ensures complete sterility the lack of infection in the controls indicates that soil sterilization was adequate to kill the mycorrhizal fungi.

Two factors may help to account for the variable results of individual inoculation experiments and the relatively low proportion (approximately 33 per cent.) of successful spore inoculations in plugged test-tubes. The variability may be partly explained by the observation that spores from different sources vary in their germination capacity. It is interesting that Godfrey (1955) has experienced a similar variability in the germination capacity of spores of *Endogone micro-* and *macrocarpa*. Regarding the relatively low degree of infectivity the experiments themselves indicate that conditions in plugged test-tubes are unfavourable for mycorrhizal development. In plugged test-tubes sporocarp infectivity varied from 25 to 60 per cent. whereas under open pot conditions the same inoculum invariably produced 100 per cent. infection. Experiments in progress suggest that more efficient lighting of the plants in plugged tubes may assist the establishment of mycorrhiza. It should also be taken into account that the establishment of symbiotic relationships under controlled conditions is known to be difficult. Some previous mycorrhizal investigations have failed because workers were unable to establish endotrophic infections in experimental plants, even when using infected roots as inoculum. Taking a wider field it is of interest to recall that the synthesis of lichens under aseptic conditions succeeds about once in 800 times. These observations may help to put the above results in their proper perspective. In so far as it has not yet been possible either to reisolate the fungus from infected plants or to obtain a pure culture of it in a soil medium, the evidence is incomplete.

ACKNOWLEDGEMENTS

I wish to record my grateful thanks to Dr. J. E. Crosse for his help and advice in the inoculation experiments, and to Mr. E. Y. Jones and Miss J. Hay who took the photographs.

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EXPLANATION OF PLATES X-XIII

Illustrating Barbara Mosse's article on *Endogone* fructifications.

PLATE X

Figs. 1 and 2. Showing the details of structure of the sporocarps.

PLATE XI

Figs. 3 *a* and 3 *b*. Structure of the mature spore.

Fig. 4. The internal projections of the resting-spore wall.

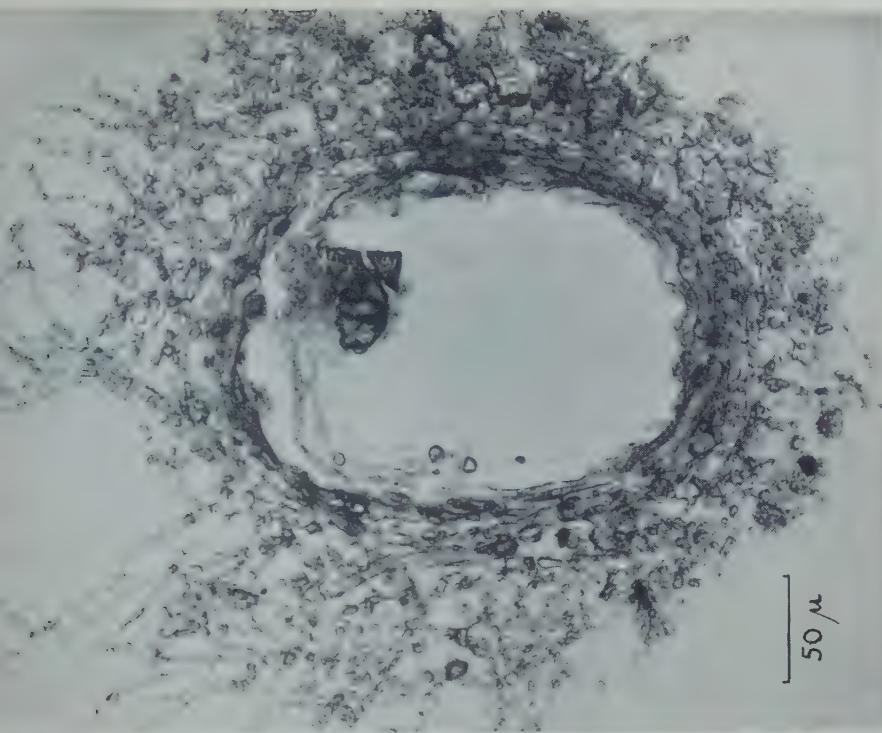
PLATE XII

Fig. 5. Apple rootlet showing mycorrhizal infection.

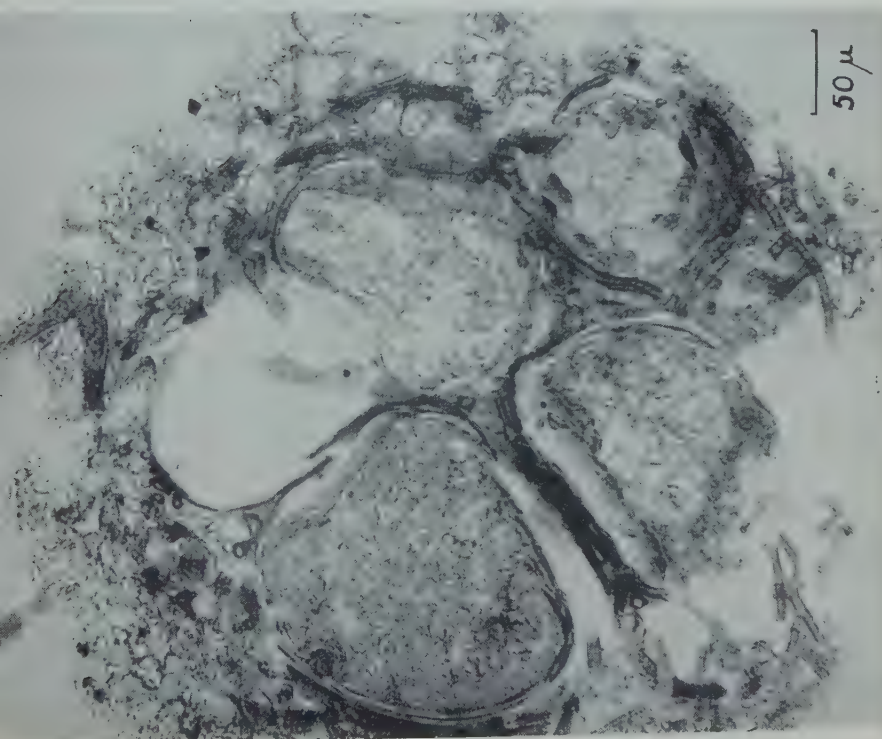
Fig. 6. Strawberry rootlets showing typical arbuscular (*a*) and endotrophic (*b*) infections.

PLATE XIII

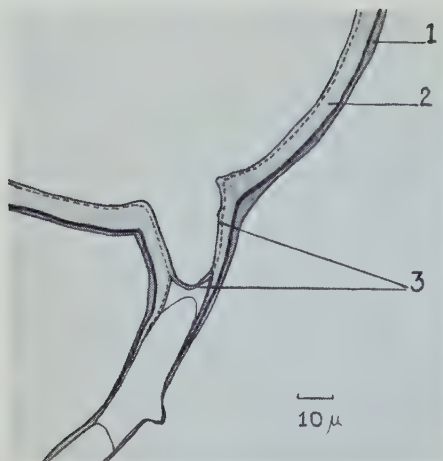
Fig. 7. Sterilized spore germinating in film of water near a strawberry root.



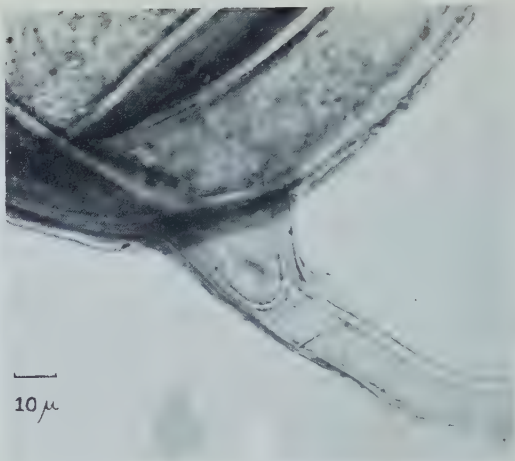
2. Section through an immature sporocarp showing an outer region of slender thin-walled hyphae, and an inner region of deeply staining thick-walled hyphae surrounding a central spore whose endospore wall is as yet undeveloped



1. Section showing the general structure of a mature sporocarp



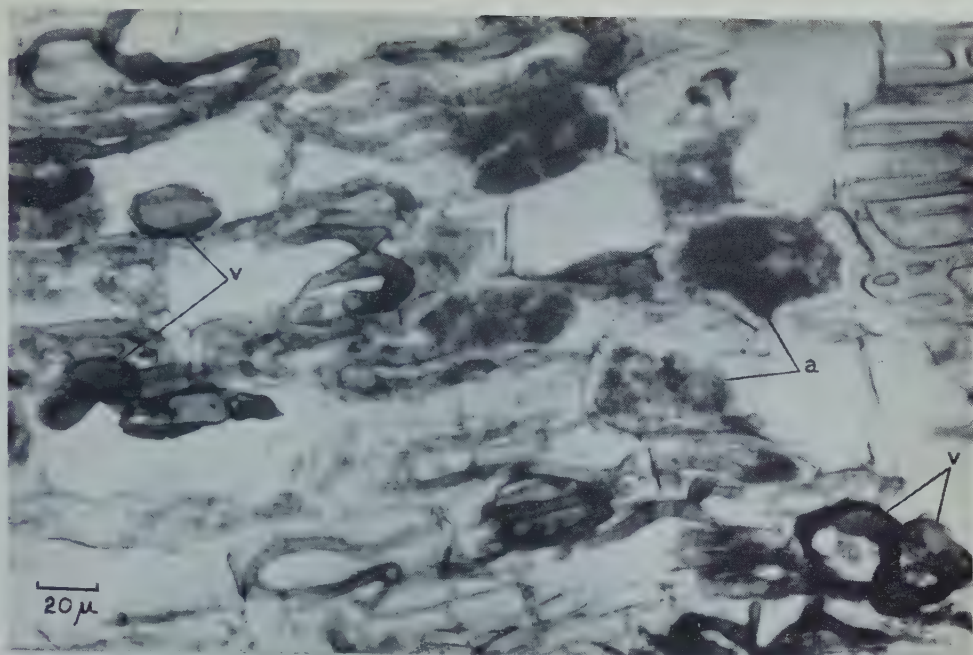
3. *a.* Diagram showing structure of a mature spore dissected from a sporocarp: 1. exospore wall, 2. endospore wall, 3. internal layer of the endospore wall (tracing of projected image)



3. *b.* Photograph of the base of the same spore



4. Detail of part of a resting spore showing internal projections arising from the endospore wall. The fine capillary opening passing through the projection and the endospore wall is indicated by an arrow. Other projections are seen in surface view with their capillary openings appearing as pin-point holes



5. L.S. of an apple rootlet showing mycorrhizal infection produced by sporocarp inoculation of autoclaved soil in open pots. Some arbuscules (a) and vesicles (v) are marked



6. L.S. of strawberry rootlets grown in plugged test-tubes in autoclaved loam inoculated with sterilized spores, and showing typical arbuscular (a) and vesicular (b) endotrophic infections



7. Sterilized spore germinated in a film of water near a strawberry root

Experimental and Analytical Studies of Pteridophytes

XXXIV. On the Shoot Apex of the Bird's Nest Fern, *Asplenium nidus* L.

BY

C. W. WARDLAW

(Department of Cryptogamic Botany, University of Manchester)

With Plate XIV and thirteen Figures in the Text

ABSTRACT

The shoot apex of the fern, *Asplenium nidus*, is described. Like a tree fern, it is unusually large but is shown to conform to a pattern common among leptosporangiate ferns. The developmental problems in these large apices are discussed.

INTRODUCTION

IN an investigation of the shoot apex of a tree fern, *Cyathea manniana* Hooker, during its ontogenetic development, the author (Wardlaw, 1948) has shown that although the apical cell and its associated meristem undergo a remarkable increase in size, their organization and the pattern of development to which they give rise persist without any fundamental change; in short, the large apical meristem of the adult plant is, approximately, a magnified replica of the small apex of a young plant. The apical meristem in ferns is thus stable and functionally efficient over a considerable size range. The progressive enlargement of the axis and of the successive leaf-bases during the development of the plant is mainly due to an increase in the amount of parenchymatous tissue in the cortex and pith. The growth of the shoot apical meristem, the sub-apical region, and the region of maturation is dependent on supplies of nutrients passing up from below: the sub-apical region, in which active cell division also takes place, shows a well-marked response to the nutrient supply—a conclusion amply supported by pure culture studies. Thus, in contrast to the distal meristem, the sub-apical and maturing regions may undergo very extensive changes during the individual development (Wardlaw, 1952). These conceptions of nutrition in relation to apical size, organization, and activity are of evident interest and importance in studies of morphogenesis. It was considered that our knowledge might be advanced by further investigations of fern species possessing large apices. To this end, apices of some very large specimens of the epiphytic fern, *Asplenium nidus* L., the bird's nest fern, have now been examined. This large and showy fern (which Copeland (1947) regards as being representative of the nest ferns) is a common epiphyte on old trees. An illustrated account of its general features, habit, and habitat in Malaya has recently been given by Holttum (1954, p. 142 et seq.). The materials used in the present investigation were collected by the writer in North Borneo in April 1952—the

region in which the photograph in Christ's 'Geographie der Farne' was taken. Chandler (1905) has described the vascular anatomy of young plants and Troll (1952) its growth-form and root system.

Recently R. and C. Wetter (1954) have discussed the relationships of the apical cell and apical meristem to the primary growth in thickness of the fern shoot. Thus, in *Polypodium glaucum*, as the individual plant increases in size, the distal region of the shoot, which is initially conical, becomes flattened (*Scheitelebene*), and eventually, on still further enlargement, is such that the apical meristem appears as a conical mound seated in a distal depression (*Scheitelgrube*). These successive changes are mainly due to the progressive augmentation of the cortical and medullary tissues. R. and C. Wetter have also indicated that there may be a direct correlation between the extent of the apical cell surface and the diameter of the shoot, but consider that fuller investigation is required. Thus, as the apical cell enlarges, it will give rise to a progressively enlarging apical meristem, the further division of the prism-shaped cells of which will result in a progressively larger sub-apical region (or *Eumeristem*) and eventually mature region. They point out, however, that the shape of the shoot apex does not stand in any strict relation to the circumference of the mature shoot; but, in general, the sunken type of apex yields a shoot of large diameter. These indications of the mechanism of the ontogenetic enlargement of the fern shoot are in general accord with the present writer's views.

MATERIALS

The materials of *A. nidus* investigated consisted of very large apices which had been preserved in 50 per cent. alcohol. These were trimmed down to manageable proportions and cut in median longitudinal and in transverse section.

OBSERVATIONS

Some of the apices were found to be in a relatively inert state, starch grains being abundantly present in the cells of the apical meristem. Other apices were in a more active condition as judged by the appearance of the apical cell; but in none was there an upgrowth of young fronds, as in the specimen illustrated by Holttum (1954). A cyclical apical activity, probably associated with the incidence of wet and dry spells, is thus apparently characteristic of this species. A comparable apical inactivity is characteristic of temperate ferns during the winter months.

When the detritus and ramenta are removed from the apex, the latter is seen to consist of a broad, shallow, saucer-like depression, about 3.0 cm. in diameter (Pl. XIV, Fig. 1). As seen with the naked eye, the centre of this saucer, occupied by the apical meristem and very young leaf primordia, is flattish, but, on the rising sides of the saucer, the closely packed, older leaf primordia, each with a greatly distended base, and a small distal region, can be distinguished, Pl. XIV, Fig. 1. *Asplenium nidus*, in short, affords a large

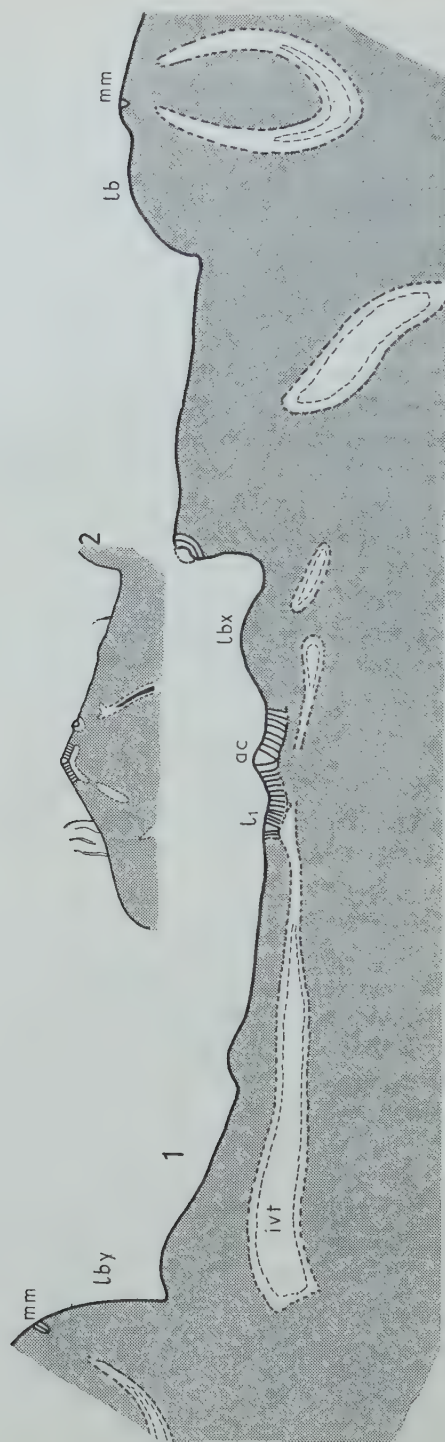
and striking example of a depressed shoot apex (*Scheitelgrube*). The phyllotaxis appears to be 5+8.

The conspicuous features of the shoot apex, as seen in longitudinal median sections, are (i) the small size of the apical cell and distal meristem relative to the diameter of the shoot, (ii) the extensive sub-apical region and subjacent region of maturation, (iii) the very wide pith immediately below the apex and the consequential almost horizontal disposition of the pre-vascular tissue, and (iv) the very extensive enlargement of the basal region of leaf primordia close to the apex (Text-figs. 1 and 3). Because of the great enlargement of the older leaf-bases, the extent of the cortex may be difficult to define.

The early development of a leaf primordium is as in other leptosporangiate ferns, i.e. it originates as a circular or elliptical group of prism-shaped cells of the apical meristem, Text-figs. 8, 10, Pl. XIV, Fig. 2. The abaxial side soon shows rapid and extensive growth, whereas the adaxial side grows relatively slowly and retains its flattish surface for some time. Farther out in the sub-apical region, the abaxial side undergoes a very conspicuous development: it becomes considerably elongated and so much enlarged that its surface is virtually at right-angles to the shoot axis, instead of being parallel to it as in smaller ferns (see Text-fig. 6). These developments become more conspicuous the farther the primordium is from the apical meristem (Text-fig. 3) and the adaxial side also shows considerable growth and bulges out. The enlarging primordium is now seen to consist of a greatly extended, vascularised, parenchymatous base supporting a relatively small, circinate curved distal region. The marginal meristems, Text-figs. 1, 9, which are formed from the lateral segments of the leaf apical cell and give rise to the tissues of the leaf, can only be traced for a relatively short distance downwards, i.e. they disappear as the extensive parenchymatous development of the leaf-base takes place.

These features of large apices of *A. nidus* may be compared with those of a small lateral shoot, Text-fig. 2, and Pl. XIV, Fig. 5, in which the conical shape of the apex is well seen. The apices of a tree fern, *Cyathea manniana*, Text-figs. 4, 12, of a large temperate fern, *Dryopteris aristata*, Text-figs. 5, 13, and of a small temperate species of *Asplenium*, *A. adiantum-nigrum*, Text-fig. 6, and Pl. XIV, Fig. 6, have also been illustrated for comparison. The apex of *C. manniana* resembles that of *A. nidus* in that it occupies a distal depression, but in the latter, although the apical cell and meristem are smaller, the pith is relatively wider. In a large apex of *D. aristata* the distal region is flattish but not depressed, and although the leaf-bases soon become enlarged, and indeed almost circular in cross-section, this development is small relative to that in *A. nidus* or *C. manniana*. The apical cells in *A. nidus* and *D. aristata* are of about the same size, both being considerably smaller than that of *C. manniana*.

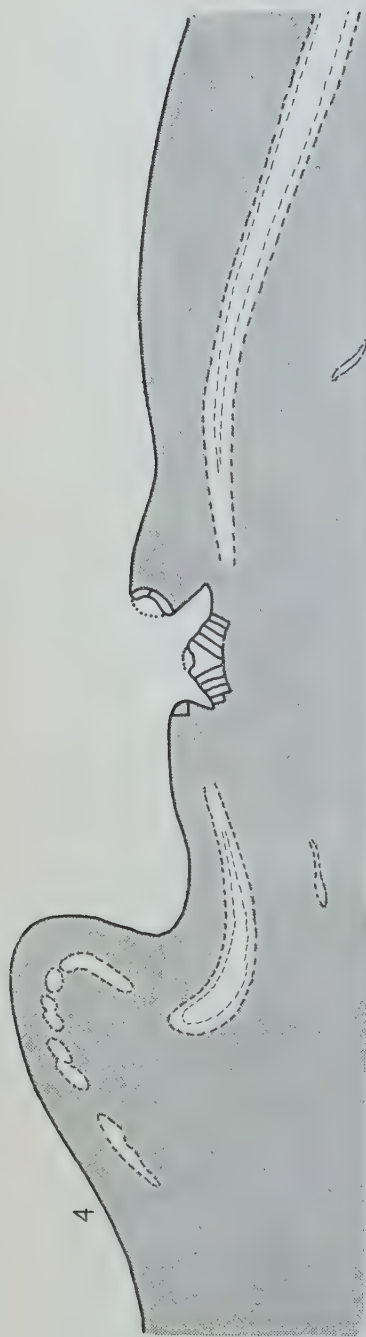
In the apex of *A. nidus* shown in Text-fig. 1, which was evidently in a resting condition, the superficial, prism-shaped cells of the apical meristem, even those close to the apical cell, have undergone a number of divisions by



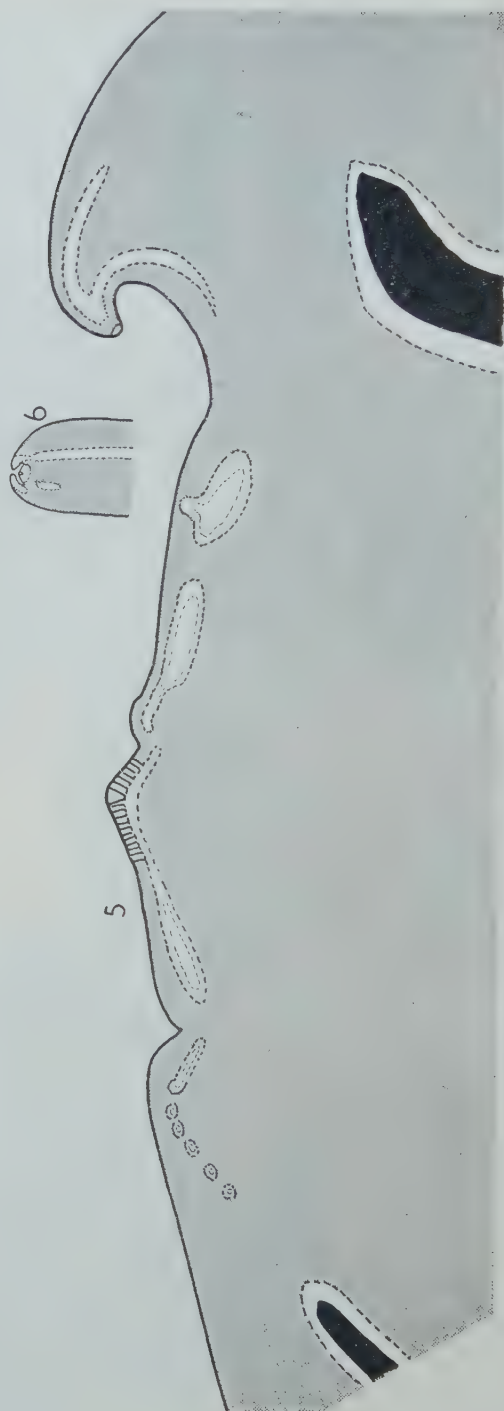
TEXT-FIGS. 1 and 2. *Asplenium nidus*. Fig. 1. Longitudinal median section of the shoot apex, showing the apical meristem, and the last-formed leaf primordium (l_1); lb , leaf-base, with leaf-trace; mm , marginal meristem of leaf; ivt , incipient vascular tissue. Fig. 2. Lateral shoot apex in l.s.



TEXT-FIG. 3. Longitudinal median sections of leaf primordia, showing the greatly enlarged basal region. The leaf primordium on the left corresponds to *lb*_y and the small primordium, *z*, to *lb*_x in Fig. 1. ($\times 10$.)

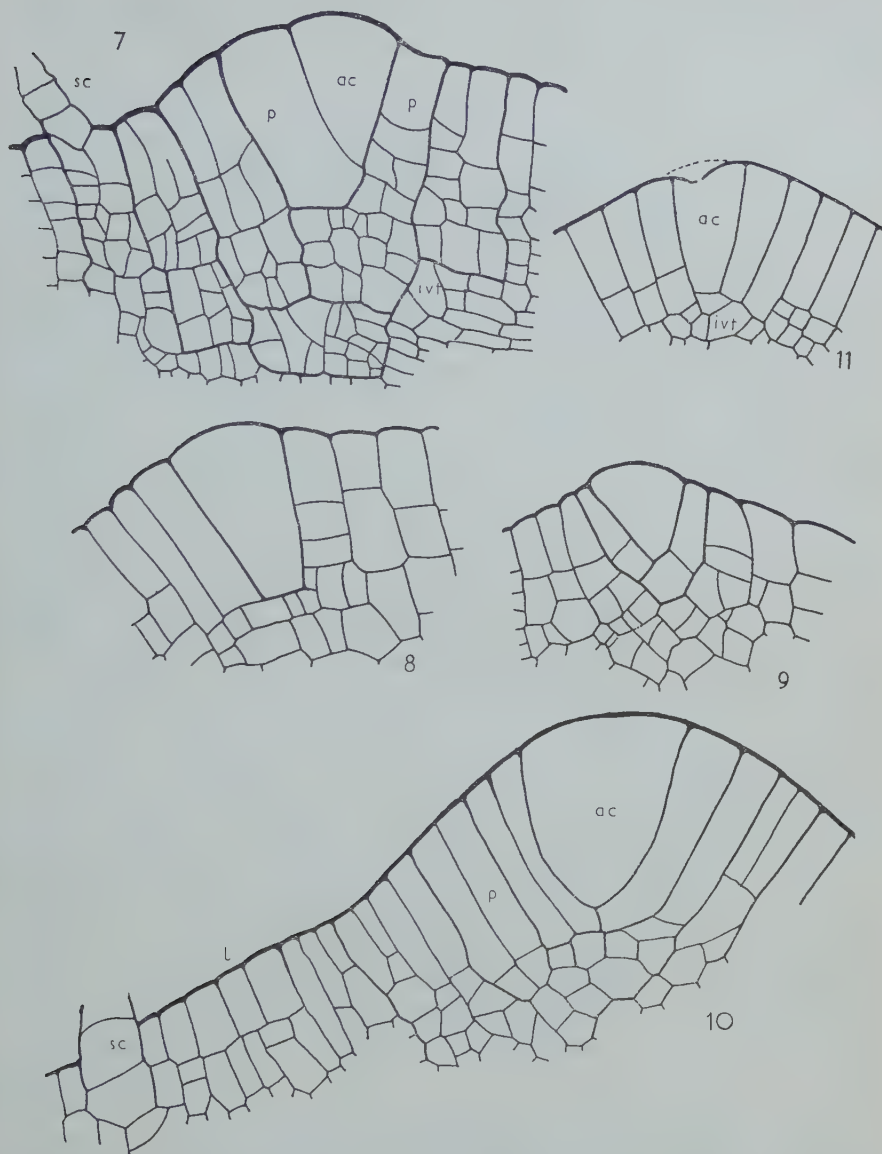


TEXT-FIG. 4. *Cyathea manniana*. Longitudinal median section of a large apex ($\times 10$).



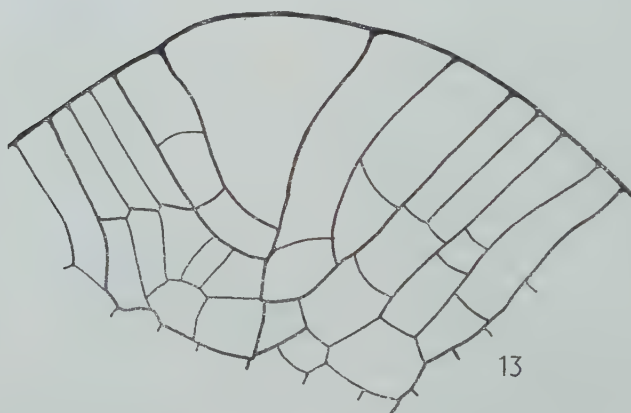
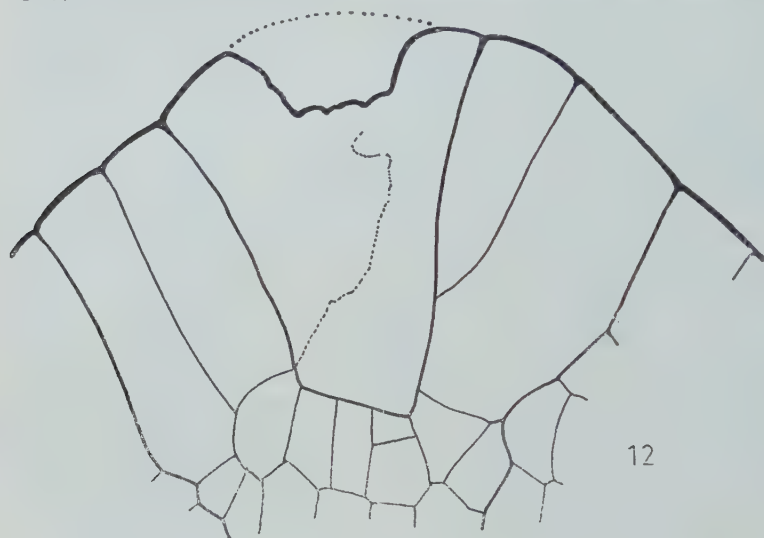
TEXT-FIG. 5. *Dryopteris aristata*. Longitudinal median section of a large apex ($\times 10$).

TEXT-FIG. 6. Longitudinal median section of the apex of *Asplenium adiantum-nigrum* ($\times 10$).



TEXT-FIGS. 7-11. *Asplenium nidus*. Fig. 7. Longitudinal median section of an inactive shoot apex, showing the apical cell (*ac*), the prism-shaped cells of the apical meristem (*p*), the incipient vascular tissue (*ivt*), and a scale (*sc*). Fig. 8. Longitudinal median section of a young leaf primordium; the shoot apex is towards the right. Fig. 9. A marginal meristem, as seen in a longitudinal section of an enlarging leaf-base; this meristem can be followed downwards for a short distance below the circinnately curved leaf apex. Fig. 10. Longitudinal median section of a more active shoot apex, showing the prism-shaped cells (*p*), and a very young leaf primordium (*l*) not cut in quite the median plane. Fig. 11. Longitudinal median section of the apex of a small lateral shoot (all $\times 180$).

periclinal walls, so that the meristem has an almost parenchymatous appearance. These cells contain abundant starch grains. Even in an inactive apex such as this, the incipient vascular tissue (or pre-vascular tissue, *ivt*, in Text-fig. 7) could be traced to a point close to the apical cell group. In the



TEXT-FIGS. 12 and 13. Longitudinal median sections of large shoot apices of *Cyathea manniana* and of *Dryopteris aristata* respectively ($\times 180$).

rather more active apex illustrated in Text-fig. 10, the apical meristem of prism-shaped cells is not only more extensive, but these cells are showing evidence of division by anticlinal walls as well as by periclinal walls close to their inner faces. As indicated by earlier investigators, by the writer, and recently by R. and C. Wetter, it is by the further division of these inner segments of the prism-shaped cells that the vascular and medullary tissues of the leptosporangiate fern axis originate. In the developing leaf-bases of

A. nidus, the extent of cell division in the cortex by anticlinal and periclinal walls is impressive.

DISCUSSION

The observations recorded above have shown that the large shoot apex in *Asplenium nidus* is comparable in all essential respects with the large apices of other leptosporangiate ferns. The conspicuous features are the formation of a very large pith immediately below the apical meristem and in the sub-apical region, and the very extensive parenchymatous enlargement of the leaf-bases, both of these contributing to the saucer-like configuration of the distal region of the shoot. While it is true, as R. and C. Wetter (1954) have pointed out, that the development of a shoot of large diameter can be related to the activity of a large apical meristem, it is apparent in this fern that further active growth and cell division, by periclinal and anticlinal walls, in the sub-apical region, account for the very extensive development of cortex, pith, and leaf-bases. This growth depends on supplies of nutrients moving acropetally by way of the vascular system from the functional leaves and older regions of the shoot below. Wardlaw (1952) and Wardlaw and Cutter (1954, 1955) have indicated that it is reasonable to infer that the incompletely differentiated vascular tissue in the apical and sub-apical regions is also effective in translocation. In these regions, the histological developments are indicative of the centrifugal and centripetal movement of nutrients (in the widest sense) from the vascular tissue into the peripheral and central tissues respectively, the supplies of nutrients diminishing and changing qualitatively as the apical cell is approached. To what extent the characteristic growth-form of this species can be referred to the ratio of carbon to nitrogen in the growing region suggests further interesting investigations. The observations which prompt this thought are (i) the presence of a large rosette of functional leaves, presumably with a considerable capacity for the synthesis of carbohydrates, and (ii) possible limitations in the supplies of mineral nutrients, especially nitrogen, in relation to the epiphytic mode of life. The production and utilization of growth-regulating substances in a species in which there is such a rapid transition from the distal embryonic region in shoot and leaf to the greatly distended subjacent parenchymatous region, also suggest problems of special interest.

During the obconical enlargement of the fern shoot there is a decrease in the ratio *area of cortex : area of pith* as observed in transverse sections (Wardlaw, 1945, 1947). In the very broad apex of *Asplenium nidus*, this value must be a low one. Even an approximate value would, however, be difficult to obtain because of the extensive development of the leaf-bases. This ratio is, as it seems, determined by the histogenic activity in the apical meristem and by the centripetal and centrifugal movement of nutrients from the pre-vascular and maturing vascular tissue. The illustrations show that the tissue pattern, and, in particular, the position of the apical meristem in a distal depression, are referable to the large radial component of growth in the axis

and leaf-bases, i.e. at right-angles to the pre-vascular and vascular tissue. The sunken apex of large shoots of *Polypodium glaucum* are likewise attributed by R. and C. Wetter to the extensive periclinal divisions in the prism-shaped cells and their derivatives.

In a geometrical analysis of the growth and histological pattern in the large shoot apex of *Microcycas calocoma*, Schuëpp (1952) has shown that the cellular pattern at the apex can be referred to a series of *displacement*, or *dislocation*, curves which intersect with *growth* curves. Thus, if one proceeds basipetally from the most distal group of cells, different points along a displacement curve will afford an indication of what a particular group of embryonic cells will look like as their age, differentiation, and maturation increase; and a point moving along such a curve will intersect the successive growth curves at equal intervals of time. These two sets of curves can be used to construct a system of *periclinal* and *anticlinal* respectively, the interstices between them increasing in size and becoming characteristically deformed, each according to its initial position, as it becomes farther and farther displaced from the apex. As the cells in a particular interstice pass from one zone to another, e.g. from a sub-apical position to one vertically lower down, or to one in a lower and more lateral position, the direction of maximum growth may change, e.g. from anticlinal to periclinal or oblique; the interstice itself, originally square, may be deformed to a rectangle, a parallelogram, or to a less regular four-sided figure; and the pattern of cell walls within the interstice will correspond to the pattern of dislocation and growth curves. These several geometrical figures, however, all 'evolve' within the same growth pattern. Schuëpp considers that the large dome-like apex of *Microcycas* may be attributed to the more rapid growth of the surface layer as compared with that of the inner periclinal, or to the extent of the radial growth relative to the tangential growth in the outer tissues. This analysis of the apex of *Microcycas* has suggested to Schuëpp 'the idea of a growth pattern being brought about by a disposition of the protoplasm-molecules similar to the interior structure of crystals'. He points to the essentially dynamic, self-regenerating, and variable nature of the pattern, the fate of each cell of which is determined by the dislocation curve along which it moves during growth. He further considers that the histological constitution of the several types of apical meristem should be 'explained by their growth patterns which may be fully represented by dislocation curves or evolution lines, growth curves, periclinal and anticlinal and their derivatives'.

This interesting analysis focuses attention on the importance in morphogenesis of the different rates and directions of growth of cells occupying different positions in the apical and sub-apical regions. Schuëpp's procedure might well be applied to large fern apices with important results. But, as it seems to the writer, in the light of the present and earlier studies of fern apices, the fundamental problem relates to the factors which determine differential growth in cells and cell groups. At least the lines along which partial solutions to this very difficult and complex problem may be sought

can be indicated. The growth of a cell, or a cell group, at the shoot apex, may be referred to: (i) the genetical constitution of the species; (ii) the position which the cell occupies; this involving, among other things, the size of the apex, and determining the incidence of such physical factors as the mutual pressure of adjacent tissues; (iii) its competence to react to growth stimuli and to utilize nutrients; (iv) the direction of supply of nutrients, their composition and concentrations; (v) the regulative effect, on cells of different reactivity, of hormonal substances; and (vi) environmental factors. Some of these matters have already been considered in earlier papers in this series. As the resultant effect of the interaction of all the factors at work in the apex is an orderly or harmonious development, it is to be expected that this will be susceptible of geometrical analysis, e.g. along the lines indicated by Schuëpp. It thus becomes increasingly evident that our understanding of the apical meristem, and of the leafy-shoot to which it gives rise, will be advanced in so far as geometrical analyses and the processes involved in growth and morphogenesis can be combined.

The writer wishes to thank Mr. E. Ashby for this assistance in photography and in the preparation of microscope slides.

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EXPLANATION OF PLATE XIV

Illustrating Professor C. W. Wardlaw's paper on the shoot apex of *Asplenium nidus*. (All figures are from untouched photographs.)

Figs. 1-5. *Asplenium Nidus*.

Fig. 1. Downward view of the shoot apex, most of the scales having been removed. The small distal and the distended basal region of the larger leaf primordia can be seen ($\times 2$).

Fig. 2. A young leaf primordium in transverse section, the shoot apex being towards the left ($\times 225$).

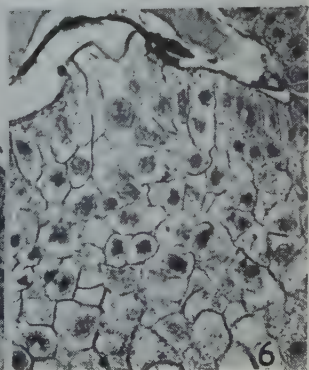
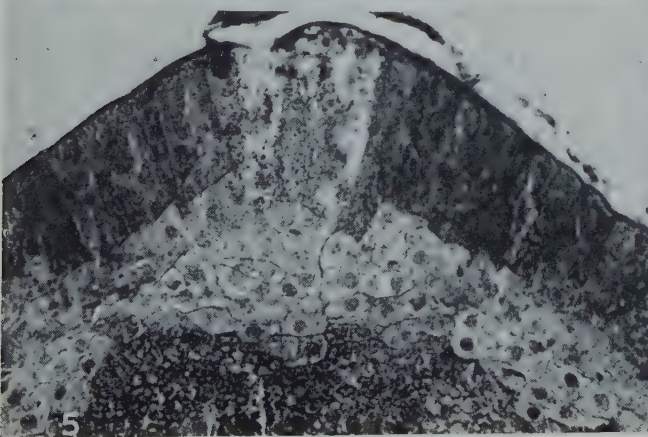
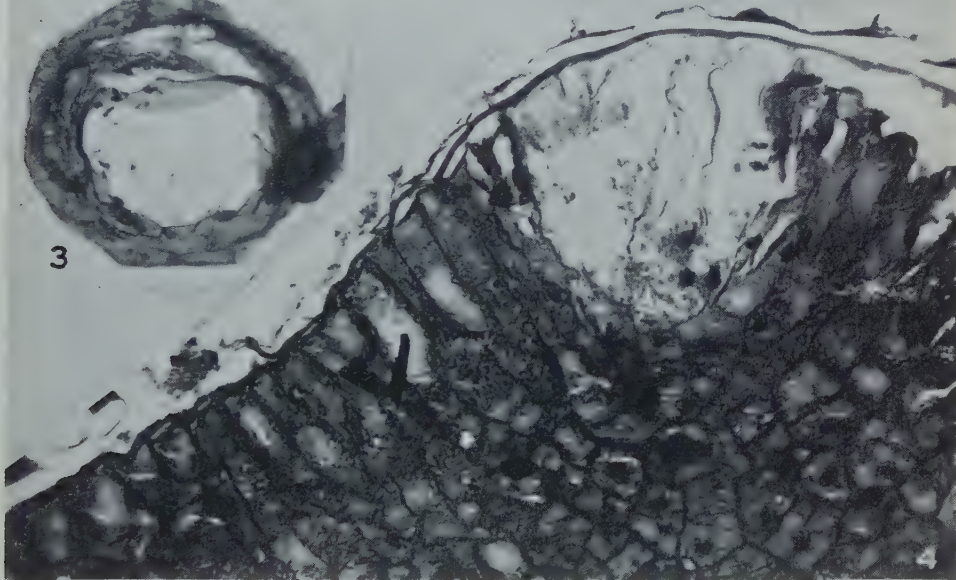
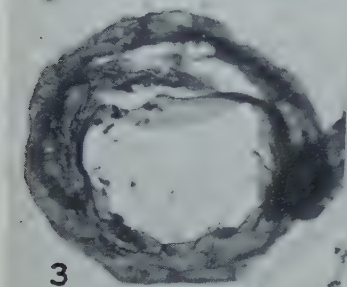
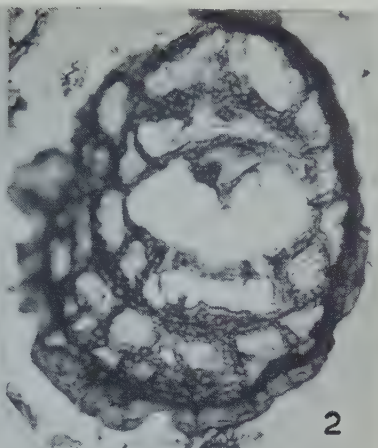
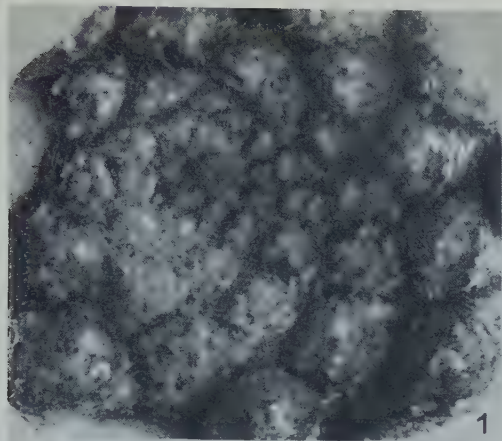
Fig. 3. Shoot apical cell in transverse section ($\times 225$).

Fig. 4. Large shoot apex in longitudinal median section (slightly oblique) showing the apical cell and prism-shaped cells of the meristem (on left) ($\times 225$).

Fig. 5. The apex of a small lateral shoot in longitudinal median section, showing the apical cell, the prism-shaped cells of the apical meristem, the underlying pre-vascular or incipient vascular tissue and the pith ($\times 225$).

Fig. 6. *Asplenium adiantum-nigrum*.

Shoot apex in longitudinal median section ($\times 225$).



A New Species of Abietaceous Cone from the Lower Greensand of the Isle of Wight

BY

G. T. CREBER

With Plate XV and five Figures in the Text

ABSTRACT

An account is given of a hitherto undescribed species of *Pityostrobus* the specimens of which come from the Lower Greensand of the Isle of Wight and for which the name *Pityostrobus jacksoni* is suggested. Structurally it has the general characters of the cones of the modern genus *Pinus* but no immediate affinity to an individual species of that genus is proposed. As a highly characteristic feature of the cone the seed-scale complexes possess much inflated apophyses, definite bract scales are also present. The information obtained from sections through various planes is embodied in a reconstruction of one seed-scale complex together with its bract scale.

DURING the last 120 years there have been many examples recorded of fossil Abietaceous cones. Lindley and Hutton (1835) described *Abies oblonga* from the Greensand of Lyme Regis and *Pinus primaeva* from the Inferior Oolite. Coemans (1866) described *Pinus corneti* and *P. andraei* from the Wealden of Belgium and further foreign specimens were recorded by Cornuel (1866) from the Wealden of the Paris Basin. Carruthers (1866, 1869) was responsible for recording some important British specimens, notably *Pinites dunkeri*, *P. leckenbyi*, and *P. defectus*; furthermore he recognized the Abietaceous nature of *P. macrocephalus* (Lindley and Hutton) and *P. sussexiensis* (Mantell) which had originally been described as species of *Zamia*. The work of Fliche (1896) and Fliche and Zeiller (1904) extended the fossil record of foreign specimens by describing several cones from France.

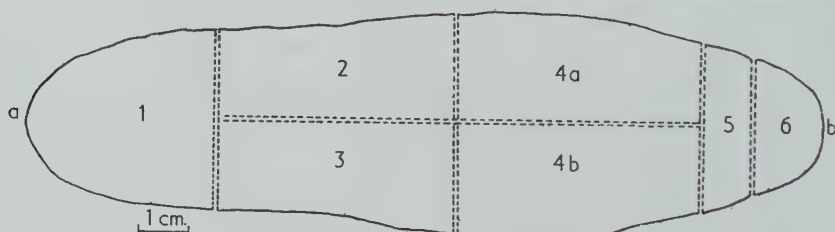
Up to this time the descriptions of cones had been based almost exclusively upon their gross external features together with an occasional median vertical section to illustrate the general arrangement of the scales. The first appearance of details of microscopical anatomy was in the full accounts of *Pino-strobus sussexiensis* and *P. benstedii* (Mantell) given by Stopes (1915). Stopes resuscitated Feistmantel's genus *Pino-strobus* for fossil cones resembling those of the modern genus *Pinus* and, further, proposed *Cedrostrobus* for those resembling the modern *Cedrus*. Seward (1917), contrary to this suggestion, strongly advocated the use of *Pityostrobus* which had originally been proposed by Nathorst (1897) for fossil cones showing general Abietaceous features. Seward accordingly transferred all previously described cones to this form-genus and the name has in fact been used exclusively since 1919. The use of a fairly broad form-genus is clearly a useful preliminary to a more precise

classification that may be possible eventually when the whole range of fossil Abietaceous cones is worked out.

Further details of microscopical anatomy were furnished by Dutt (1916) who investigated *P. macrocephalus* with special reference to the seeds. The most recent work in this field is that of Alvin (1953) who made a comprehensive study of *P. andraei* (Coemans), *P. bommeri* Alvin, and *P. corneti* (Coemans). The specimens which form the basis for the present study are calcareous petrifications collected from the Lower Greensand at Shanklin in the Isle of Wight and now held in the Collection of the Geology Department of the British Museum (Natural History). The diagnosis of the new species is founded on the main specimen, a long cylindrical cone collected by Mr. J. F. Jackson, sometime curator of the Sandown Museum. The two subsidiary specimens, V. 18858 and V. 21732, are globose in shape, but an investigation of their anatomy shows them clearly to be much abraded fragments of long cylindrical cones. As they resemble the main specimen in every detail except one, which will be discussed later, they are being considered as con-specific.

DESCRIPTION OF THE CONE

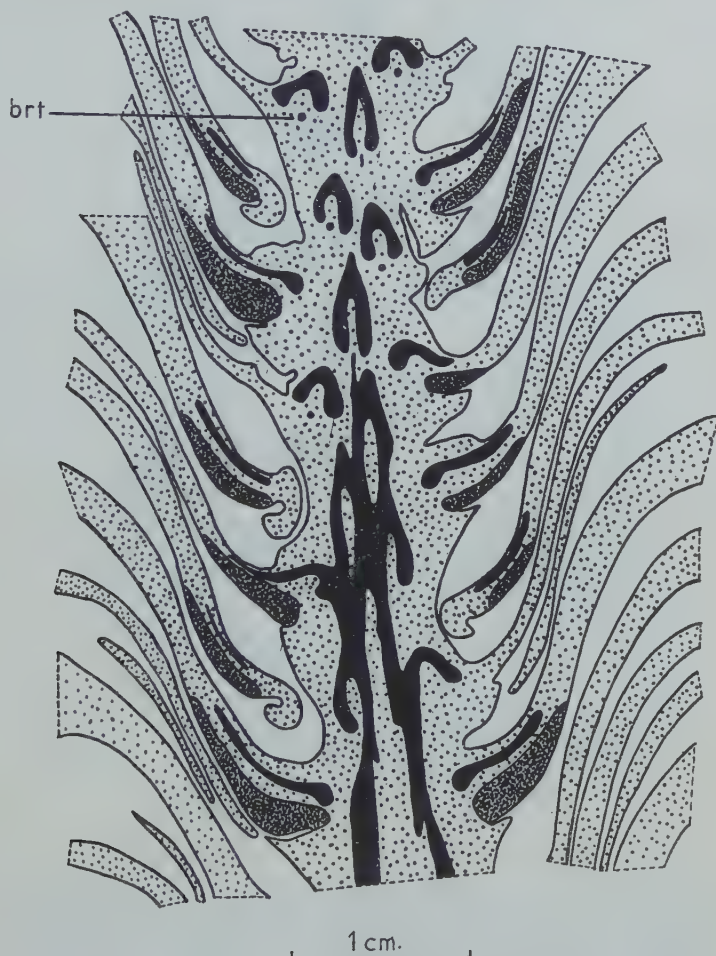
At the commencement of the present investigation the main specimen had already been cut into blocks (Text-fig. 1). A longitudinal rock section had



TEXT-FIG. 1. A drawing of the outline of the specimen showing the blocks into which it was cut. *a*, apex; *b*, base.

been made from material between blocks 2 and 3; there were also four transverse rock sections. So as to measure the specimen in a state approximating to the original, plasticine was inserted between the slit surfaces to compensate for the loss of material in making the rock sections, and when this had been done the length was found to be 15.6 cm., the breadth at the widest part 4.4 cm. and at the narrowest part 3.7 cm. Direct inspection of the hand specimen furnished no estimate of the original morphology of the external surface of the cone as the apophyses of the seed-scales had been lost by abrasion in the distal portion of the cone and were totally imbedded in matrix in the proximal portion. By cutting block 4, however, into blocks 4*a* and 4*b* it was then possible to see the form of the apophyses in longitudinal section. As the cone had been petrified after dehiscence no seeds were observed at all in the specimen.

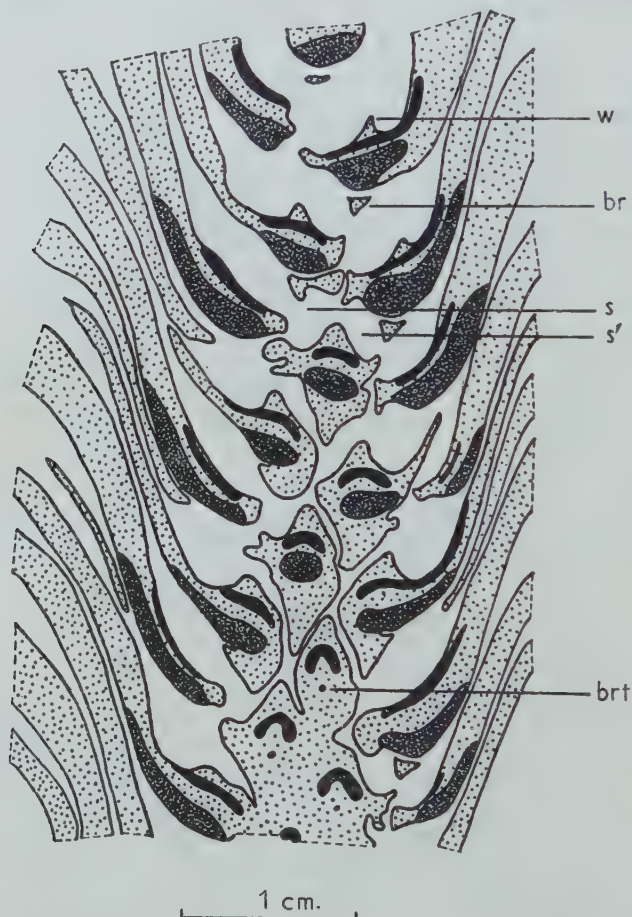
The diameter of the axis is about 9 mm. and is relatively uniform throughout much of the length of the cone. In transverse section (Text-fig. 4) the bases of the seed-scales and bract-scales appear as broad protuberances. The axial vascular cylinder, broken by 'leaf-gaps' opposite the scales, encloses a pith 3 mm. in diameter composed of uniform parenchymatous cells. The



TEXT-FIG. 2. A drawing of a tangential longitudinal section through the cone axis. Xylem is represented by the black areas, sclerenchyma is densely stippled, and other tissues are sparsely stippled. *brt*, bract trace.

regions of primary xylem are made more obvious by the presence near each of a resin canal. The phloem is not well preserved, it is largely represented by regions of decay which have become secondarily filled with iron pyrites. The origin of the vascular supply to each seed-scale complex can be followed from Text-fig. 2 which was drawn from a 'peel' obtained from the surface of block 3. The plane of section inclines from the radial longitudinal at the base

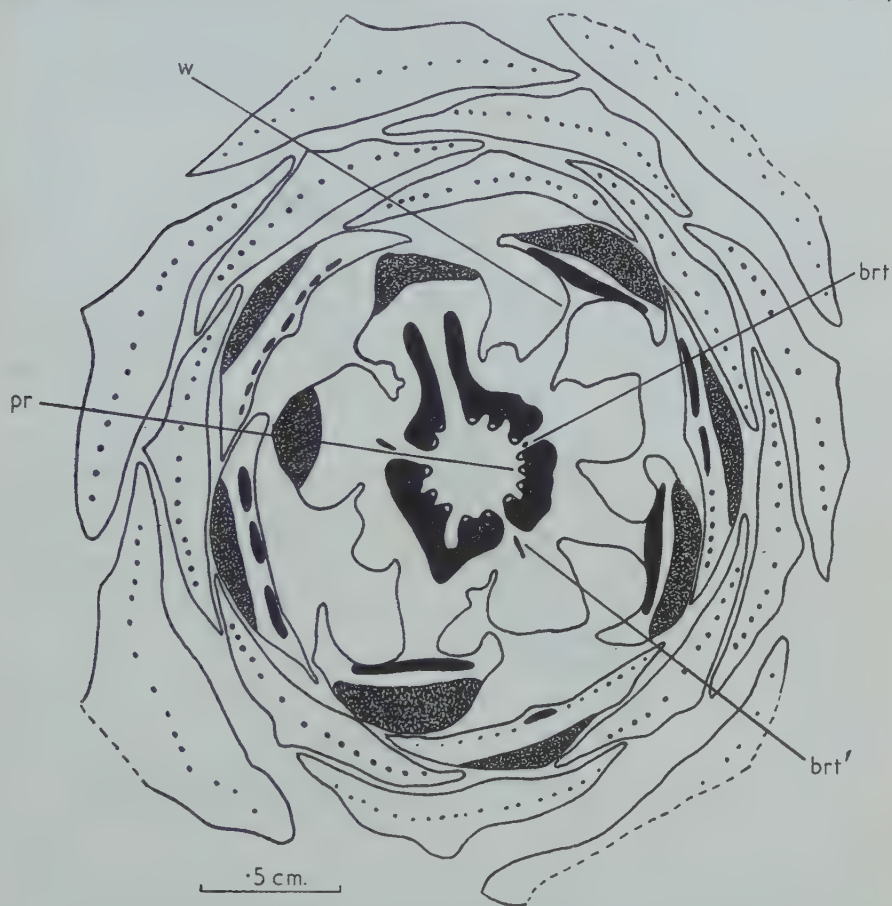
to the tangential longitudinal at the top; the surface of block 3 had been left in this state after material had been removed to make a rock section. It does, however, serve to show the change of form that the seed-scale vascular supply undergoes as it leaves the stele. Oval gaps first appear in the cylindrical stele



TEXT-FIG. 3. A drawing of a tangential longitudinal section through the cone in the region where the seed-scale complexes are attached to the axis. Xylem is represented by the black areas, sclerenchyma is densely stippled, and other tissues are sparsely stippled. *br*, bract-scale; *brt*, bract trace; *w*, wedge of tissue that separated the seeds; *s*, *s'*, spaces left by the seeds.

and then the traces assume the characteristic 'inverted horseshoe' shape with the bract traces just below the open arms of the 'horseshoes' (Pl. XV, Fig. 1). The further progress of the vascular supply to the seed-scales and the eventual separation of the seed-scales from the axis is shown in Text-fig. 3. This was drawn from a 'peel' obtained after considerable grinding of block 2, it is somewhat oblique and therefore shows the gradual stages of separation. The traces of the seed-scales become broad flat strands lying close to the

adaxial surfaces of the scales; a few bract traces are also shown, but these are no longer evident once the seed-scales and bract-scales have completely separated from the axis. The bract trace commences its separate existence as a small upgrowth from one of the oval gaps in the stele (Pl. XV, Fig. 2),



TEXT-FIG. 4. A drawing of a transverse section of the cone; the outermost seed-scales are incomplete owing to abrasion. Xylem is represented by the black areas and the sclerenchyma is densely stippled. *brt*, a bract trace cut transversely; *brt'*, a bract trace cut longitudinally; *pr*, primary xylem with resin canal; *w*, wedge of tissue that separated the seeds.

and after passing obliquely upwards for a short distance through the cortex, bends down towards the bract-scale (Text-fig. 4).

The seed-scales leave the axis almost at right angles (Pl. XV, Fig. 4) and then after bending abruptly each has a long straight portion which finally terminates in a much inflated apophysis. The base of each seed-scale complex is supported by a thick zone of sclerenchyma in which run several resin canals (Pl. XV, Fig. 3). This zone of sclerenchyma is separated from the broad

strand by a region of parenchyma; in the subsidiary specimen V. 21732 the resin canals run in this parenchyma and this constitutes the one point of difference, previously mentioned, between the main and subsidiary specimens. In the long straight portion of the seed-scale the shape becomes thin and laminoid and the vascular supply gradually subdivides into many small rounded bundles (Text-fig. 4). The bract-scales are short apiculate scales about 4 mm. long in the axils of which arise the seed-scale complexes. There



TEXT-FIG. 5. A reconstruction of one seed-scale complex. *br*, bract-scale; *a*, *b*, *c*, *d*, *e*, the major sections on which the reconstruction was based.

is a rhomboidal zone of attachment where each seed-scale complex and its bract-scale are joined to the axis (Text-fig. 3); it is not until a point farther from the axis is reached that the two become separate entities, thus showing that there is some slight degree of fusion between them. The upper point of the rhomboidal zone of attachment becomes prolonged into a wedge of tissue that originally separated the seeds (Text-figs. 3 and 4).

A reconstruction of one seed-scale complex and its bract-scale is presented in Text-fig. 5. The information used in reconstructing the inflated apophysis was obtained by grinding and taking 'peels' at 1 mm. intervals from an external tangential surface of block 4*b*; it was thus possible to pass right through one complete apophysis from the umbo to the thin vertical part of the seed-scale. The shape of the rest of the seed-scale was obtained from the various rock sections, and also the numerous 'peels' that were taken during the investigation.

DISCUSSION

The present species is of interest not only because the detailed anatomy of a similar cone has not yet been recorded, but also because its type seems somewhat uncommon among those fossil cones whose external features only have been described in the literature. In fact it would appear that by far the commonest type of Abietaceous cone in the early Cretaceous was one possessing more or less flat seed-scales as seen on the external surface. Seward (1917) particularly stresses this point after reviewing the characteristic features of *Pityostrobus dejectus*, *P. strobiformis* (Fliche and Zeiller), *P. dunkeri*, *P. leckenbyi*, and *P. sussexiensis*. The present species with inflated apophyses to the seed-scales stands apart from these examples; a transition form may be seen in *Pityostrobus andraei*, as described by Alvin (1953), where the apophyses to the seed-scales are somewhat inflated. As regards a possible comparison with a living Abietaceous genus it would appear that the closest affinity that can be suggested is with *Pinus*. The thick type of seed-scale with the reflexed tip occurs only in this genus and the presence of resin canals, on the abaxial side only, at the base of the seed-scale is a feature which is not found in any other Abietaceous genus. Whilst an affinity such as this readily presents itself, difficulties arise in attempting to establish a closer resemblance to a subdivision of the genus *Pinus*. Shaw (1914) gave an account of the two sections of the genus, *Haploxyylon* and *Diploxyylon*; the former being the 'soft' pines with *P. strobus* L. and *P. peuce* Grisebach as typical examples, the latter being the 'hard' pines with *P. pinaster* Aiton and *P. sylvestris* L. as typical examples. In the *Haploxyylon* section the shape of the cone is cylindrical with the seed-scales usually straight and thin and only moderately sclerotic; in the *Diploxyylon* section the cone tapers to a point from a broader base and the seed-scales are strongly reflexed and very sclerotic. It seems, therefore, that the present species has seed-scales typical of members of the *Diploxyylon* section and yet the shape of the cone is of the *Haploxyylon* type. This apparent 'blending' of modern specific or sub-generic characters in a fossil species is very common and Alvin (1953) noted an extension of this principle in that he found in *Pityostrobus corneti* a mixture not merely of the specific characters of one genus but of the features of three complete genera, *Abies*, *Cedrus*, and *Keteleeria*.

In conclusion it may be stated that the present species has fairly obvious relationships with the modern genus *Pinus*, but it would be clearly undesirable to press for a direct affinity with an individual species. It is suggested that the cone should be assigned to the form-genus *Pityostrobus* and that a new species, *Pityostrobus jacksoni*, should be instituted for its reception. The diagnosis for the new species is as follows:

PITYOSTROBUS—Nathorst

Fossil cones showing general Abietaceous features.

Pityostrobus jacksoni—Sp. nov.

Cylindrical Abietaceous cone, 15.6 cm. long, 3.7–4.4 cm. in breadth. Seed-scales, spirally arranged, 3 cm. long and 1.75 cm. in breadth at the widest part, tips of seed-scales reflexed forming inflated apophyses. Bract-scales 4 mm. long showing a slight degree of fusion to the seed-scales. Axis 9 mm. in diameter, remarkably constant for the greater part of the length of the cone. Vascular supply to the seed-scale leaving the oval gap in the axial cylinder as a horseshoe-shaped xylem strand which flattens at the base of the seed-scale and eventually divides to form about 15 vascular strands which cease before the distal portion is reached. A small vascular strand arises from the bottom of the oval gap to supply the bract-scale. Sclerenchyma developed considerably on the abaxial side of the base of the seed-scale. Resin canals in the cortex of the axis and round the edge of the pith associated with the primary xylem; canals confined to the side abaxial to the vascular strand at the base of the seed-scale and distributed throughout the ground tissue at higher levels.

Type specimen: Cone with rock sections and mounted 'peels' in the Collection of the Department of Geology, British Museum (Natural History).

Horizon: Lower Greensand.

Locality: Shanklin, Isle of Wight.

Other specimens: Two cones, V. 21732 with rock sections and mounted 'peels' and V. 18858 with mounted 'peels' only, both in the Collection of the Department of Geology, British Museum (Natural History).

Horizon: Lower Greensand.

Locality: Shanklin, Isle of Wight.

ACKNOWLEDGEMENTS

Grateful acknowledgements are due to Mr. W. N. Edwards, Keeper of the Department of Geology, British Museum (Natural History), for the loan of the specimens on which the investigation was carried out; to Mr. P. R. Bell of University College, London, and to Dr. K. L. Alvin of Birkbeck College for many helpful discussions during the progress of the work.

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EXPLANATION OF PLATE XV

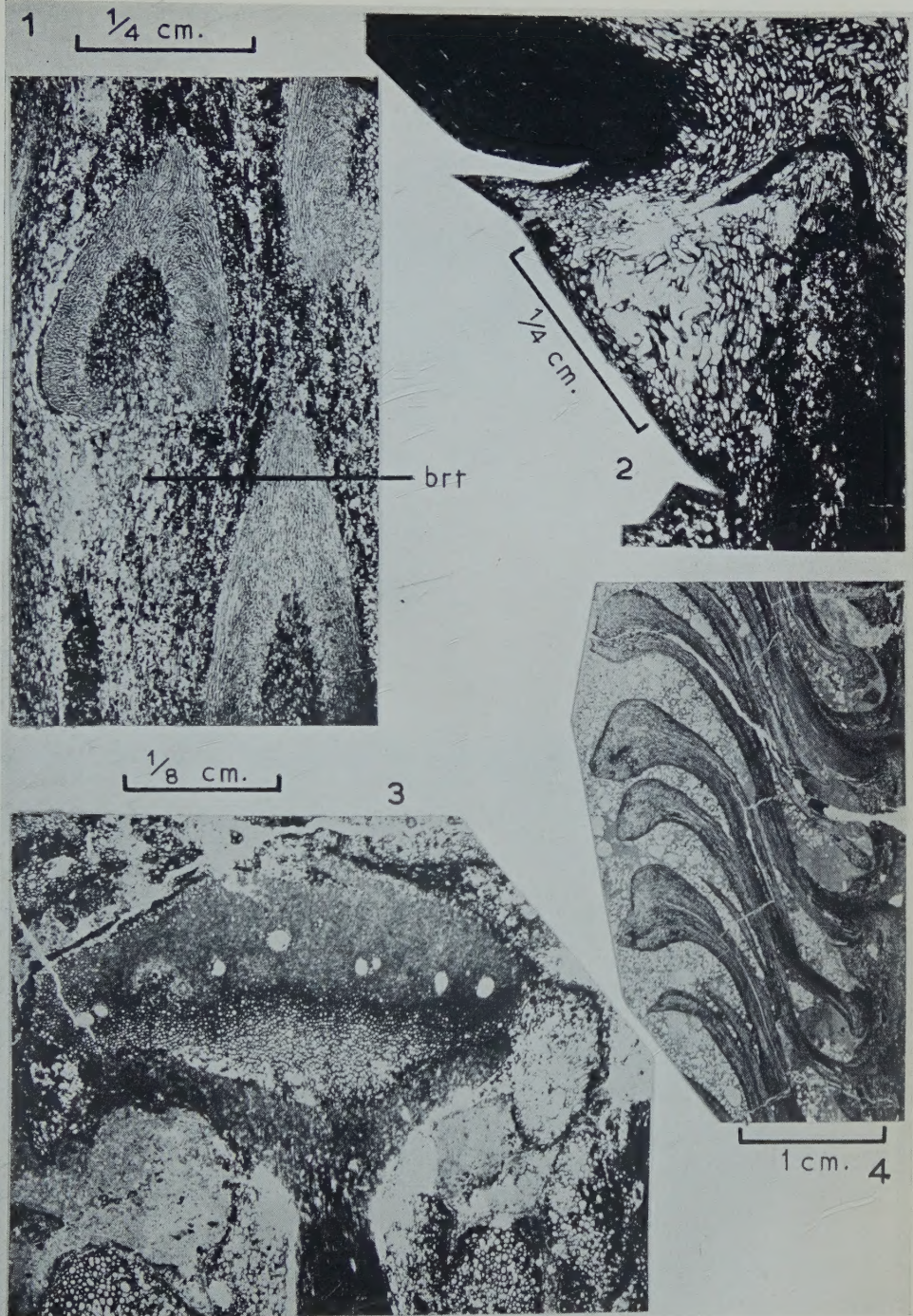
Illustrating G. T. Creber's article on 'A New Species of Abietaceous Cone from the Lower Greensand of the Isle of Wight'.

Fig. 1. Part of a tangential longitudinal section of the cone axis. One complete seed-scale trace is shown together with parts of two others. *brt*, bract trace.

Fig. 2. Longitudinal section of one bract-scale and its vascular supply. The black area above the bract-scale is the zone of sclerenchyma at the base of the axillary seed-scale complex.

Fig. 3. Transverse section of the base of one seed-scale. The resin canals are clearly seen in the sclerenchyma; the latter is separated from the strip of vascular tissue by a zone of parenchyma.

Fig. 4. Radial longitudinal section of the cone showing several seed-scales and their bract-scales attached to the axis.



G. T. CREBER

